

An examination of the ergogenic and adaptogenic role of dietary nucleotide supplementation upon sprint performance, immunity, gastrointestinal function, and skeletal muscle growth.

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[Broken red line] a threshold that represents the combined capacity of current nucleotide supply mechanisms to upregulate their activity and meet FND. In such a scenario additional exogenous nucleotides aren't required. Alternatively, when training stress load increases transitioning from an adaptation to maladaptation (high stress) model the NCET is breached, endogenous + dietary + functional reserve capacity pathways cannot supply sufficient nucleotide(s) relative to imposed FND [Broken Purple Line] and a Conditional Essentiality Functional Reserve Gap opens. Under high stress situations this nucleotide deficit can only be met through increasing inputs from exogenous sources either dietary and/or supplemental.

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List of Abbreviations

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
AMPK	AMP-Activated Protein Kinase
ANOVA	Analysis of Variance
APC	Antigen-presenting Cells
ATP	Adenosine Triphosphate
CDP	Cytidine Diphosphate
CHS	Contact Hypersensitivity
CMP	Cytidine Monophosphate
C ₂ C ₁₂	Well-established Mouse Myoblast Cell Line
DCP	Diphencyprone
DM	Differentiation media
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTH	Delayed Type Cutaneous Hypersensitivity
DPCP	Diphenylcyclopropenone
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
FI	Fatigue Index
GI	Gastrointestinal
GMP	Guanosine Monophosphate
HR	Heart Rate
HPLC	High-Performance Liquid Chromatography
IBS	Irritable bowel disease
IFABP	Intestinal Fatty Acid Binding Protein
IMP	Inosine Monophosphate
La	Blood Lactate
LR	Lactulose: Rhamnose Ratio
RAST	Repeated Anaerobic Sprint Test
RNA	Ribonucleic Acid
RPE	Rating of Perceived Exertion
RSA	Repeated Sprint Ability
Salivary IgA	Salivary Immunoglobulin-A
SD	Standard Deviation
ST	Sprint Times
TC	Thermal Comfort
TJP	Tight Junction Proteins
TNF- α	Tumour Necrosis Factor-Alpha
UDP	Uridine Diphosphate
UMP	Uridine Monophosphate
20-MST	20-meter multistage shuttle

Abstract

Nucleotides are low-molecular-weight intracellular compounds integral to the normal functioning of many physiological and biochemical pathways. They exert critical roles in cell division, cell repair and growth, energy metabolism, immune function, and gastrointestinal health. Structurally, they are made up of three components: (1) a nitrogenous heterocyclic base derivative of either a pyrimidine or purine (2) a pentose (deoxyribose or ribose), and (3) one or more phosphate groups. In humans the rate of nucleotide turnover is generally high, requiring endogenous replenishment via specific *de novo* synthesis and cellular salvage pathways working together to meet daily requirements. The general view expressed in the literature is that in healthy adults consuming a normal omnivorous diet exogenous supplementation of nucleotides is generally not required since dietary, *de novo* synthesis and salvage pathways are usually adequate to meet the daily nucleotide turnover requirements. However, critical exceptions exist; where nutrition is sub-optimal to meet the macro and micronutrient requirements imposed or where a clinical/physiological trauma or high-level physiological stress is present a case for exogenous nucleotide supplementation may exist. In such scenarios the transitioning of dietary nucleotides from being considered non-essential to essential becomes an important consideration. This thesis's aim was to examine the ergogenic and adaptogenic role of exogenous dietary nucleotide supplementation under the situation of supra-maximal high intensity exercise performance across a range of *in vivo* and *in vitro* physiological systems.

Dietary nucleotides are central to energy metabolism in purine and pyrimidine nucleotide pathways, also acting as regulatory and co-factors in mitochondrial electron transfer. Chapter 2 examined the potential ergogenic role of dietary nucleotides on high intensity sprint and repeated sprint performance. Principal findings from a range of matched pair, cross sectional, and repeated measures interventions implemented with varying concentrations of exogenous nucleotide supplementation from low to high dose, administered for short to long term periods indicated no significant ergogenic effect on sprint and repeated sprint performance or associated physiological indices were evident. Whilst no ergogenic effects were detected literature supports a possible adaptogenic role for nucleotide particularly in the GI system. Gastrointestinal enterocytes present an elevated level of cellular differentiation and proliferation turning over approximately every 72 hours, and as such present a high-level requirement for nucleotide to facilitate process. In addition, exercise stress may mediate increased GI distress and GI damage and injury. In chapter 3 the efficacy of nucleotides

regarding their role in ameliorating the observed effects of exercise stress on the integrity of the gastrointestinal mucosa was considered. Using a repeated measured design the permeability of the GI tract to lactulose and L-Rhamnose a probe designed to assess gut permeability was determined. Findings indicate that supplementation of nucleotides at three dose level for 4 weeks resulted in no significant difference in gastrointestinal permeability relative to placebo as a biomarker of gut integrity. It is concluded that no significant adaptogenic effect of exogenous nucleotides upon gut permeability/damage following acute high intensity exercise performance was apparent.

Undertaking periods of intense athletic training is known to impair the normal functioning of the immune system in athletes when the level of recovery is impaired. High intensity may result in cellular and humoral immune function suppression. Nucleotides in animals, pediatric and adult clinical nutrition are known to support in vitro markers of immune function, improve animal population health and survival. In chapter 4 a novel in vivo contact hypersensitivity immune challenge via diphenylcyclopropenone (DPCP) as an in vivo assessment of immune function was undertaken following 4 weeks dietary supplementation in a matched design healthy athletic male population. Primary outcome measures of erythema and hypersensitivity induration responses indicate no significant enhancement of in vivo immune function following acute high intensity exercise performance. Finally, given the central role nucleotides play in cellular division, growth, and repair the efficacy of a high and low dose of exogenous nucleotide supplementation on in vitro C₂C₁₂ skeletal muscle myoblast morphological and transcriptional change under a pro-inflammatory cytokine TNF- α model of cell damage was undertaken. Data indicates that whilst there were differential changes in gene expression, no significant effect on either cell morphological development or attenuation of myotube atrophy were present in the nucleotide supplemented conditions. In conclusion this thesis has considered the application of exogenous dietary nucleotides under a high intensity exercise stress model during both in vivo human and in vitro cell models to examine possible ergogenic and adaptogenic effects. No significant impact on exercise performance, immune function, gastrointestinal permeability, and skeletal muscle morphological and transcription responses were noted. There would appear no clear application of nucleotides in a high intensity repeated sprint exercise model as used herein. Future work should consider the efficacy of exogenous nucleotide supplementation under modes of more sustained and prolonged exercise/physiological stress such as that achieved during high level endurance exercise performance and training.

Author's Declaration

I declare that the work in this thesis was carried out in accordance with the regulations of Liverpool John Moores University. Apart from the help and advice acknowledged, the work within was solely completed and carried out by the author.

Any views expressed in this thesis are those of the author and in no way represent those of Liverpool John Moores University and the School of Sport and Exercise Science.

This thesis has not been presented to any other University for examination either in the United Kingdom or overseas. No portion of the work referred to in this research project has been submitted in support of an application for another degree or qualification of this or any other university or institute of learning.

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Wong Fui Yen

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Dedication

I dedicate this thesis to my parents and sisters, for all you have done and all you continue to do for me.

Thank you for supporting me in whatever I choose to do and for always believing in me.

Publications, Conference Proceedings and Presented Abstracts arising from this thesis:

Oral Presentation

Fui Y. Wong, Samuel Morris, Adam P. Sharples, David A. Low, Mark A. Scott, Dominic A. Doran. Nucleotide supplementation does not improve repeated sprint performance. *Journal of Sports Sciences*, 33: sup1, s5-s8. Presented at BASES Conference, Burton upon Trent (St George's Park), 25 November 2015.

Poster Presentation

Fui Y. Wong, Samuel Morris, Adam P. Sharples, David A. Low, Mark A. Scott, Dominic A. Doran. Effect of 30-days oral purine and pyrimidine nucleotide supplementation upon repeated sprint ability performance. Presented at BASES Student Conference, Liverpool John Moores University, Liverpool, 31 March 2015.

Chapter 1

General Introduction

This chapter provides a broad overview of the study conducted in this thesis and describes the research methodology applied. This chapter also covers the background of the role of nucleotide supplementation and its potential for enhanced performance in several systems, such as gastrointestinal impacts, immunological function, cell metabolism, cell proliferation and growth, as demonstrated in animal models.

1.1 Introduction

Nucleotides are organic low-molecular-weight intracellular compounds that are essential to and integral to the normal functioning of numerous physiological and biochemical pathways. They exert critical roles in cell division, cell repair and growth, inter and intracellular signalling pathways, neurotransmission, energy metabolism, immune function, and gastrointestinal health (Grimble and Westwood, 2001; Gil, 2002; Inoue et al., 2007; Sauer et al., 2012; Che et al., 2016; Kumari and Tripathi 2021). There are five different nucleotides, and each is comprised of a base, a sugar, and a phosphate group. Where the phosphate group is absent, they are designated as a nucleoside. The five nucleotides fall into two groups: Pyrimidines (Cytidine ‘C’, Thymidine ‘T’, Uridine ‘U’), and Purines (Guanosine ‘G’, Adenosine ‘A’) (Cosgrove, 1998). They are synthesised endogenously through distinct purine and pyrimidine de novo pathway using small precursors (nucleobases) and a salvage pathways using nucleosides and nucleobases recovered from the breakdown of nucleotides to resynthesize nucleic acids. Nucleotides are also obtained exogenously through the diet. They are required for many physiological/biological functions, including their critical role as the building blocks in the synthesis pathways of Deoxyribose Nucleic Acids (DNA) and Ribose Nucleic Acids (RNA) necessary for cell proliferation and differentiation (Moffatt and Ashihara, 2002; Nelson and Cox, 2014; Bowater and Gates, 2015). Nucleotides and their precursors are therefore critical to cellular growth, development and repair particularly where cells are subject to rapid turnover i.e., gastrointestinal tract, or subject to oxidative stress, the need for synthesis and replacement of high-energy cellular substrates and as co-factors/enzymes e.g., nicotinamide adenine dinucleotides (NAD) and Flavin adenine dinucleotides (FAD), as well as maintaining and modulating immune function (Carver, 1996; Nelson and Cox 2000, Grimble and Westwood, 2001; Gil, 2002; Nakamura et al., 2012). More recently their importance in microbiome modulation as part of their gastrointestinal function has been established (Martinez-Puig et al., 2007; Weaver and Kim, 2014; Waititu et al., 2017; Valini et al., 2021).

Considering the diverse biological roles’ nucleotides fulfil, whole-body nucleotide requirements and turnover is generally very high with pyrimidine nucleotide turnover in the region of 450-740 mg per day, requiring endogenous and exogenous replenishment mediated through dietary, de novo synthesis and salvage pathways respectively to maintain function (Moffatt and Ashihara, 2002; Bowater and Gates, 2015). Purine nucleotide pool turnover is

significantly larger; for example, a mole of adenosine triphosphate (ATP) is resynthesised approximately 1500 times per day with up to ~60kg of ATP resynthesised per day, where exercise is considered, this rate increases (Buno and Kolkhurst, 2021). Purine and pyrimidine nucleotide salvage pathways are highly efficient recovering approximately 95% of the body's nucleotide pool (Murray et al., 1970; Murray, 1971). However, nucleotide pool losses do occur and have been estimated to be approximately 5% of total whole-body turnover corresponding to 1280 mg of nucleotide or ~ 450 mg of purine base (about 6 mg of base per kg body weight) per day (Murray et al., 1970; Murray, 1971). As such, significant nucleotide recovery and/or replacement is needed to maintain cellular nucleotide pools and thus biological function (Hess and Greenberg, 2012). These pathways of nucleotide recovery and (re)synthesis are important with salvage pathways more energy-efficient and preferentially activated than de novo biosynthesis pathways, which require 5 or 6 moles of ATP for each mole of pyrimidine or purine nucleotide produced, respectively. During periods of rapid cell division and proliferation the relative balance between pathways becomes energetically biologically significant (Sancho-Pozo and Gil, 2002; Lane and Fan, 2015; Pedley et al., 2017).

Nucleotides are present as free nucleotides and nucleic acids in all foods of animal and plant origin (Rudolph et al., 1990; Moffatt and Ashihara, 2002; Hess and Greenberg, 2012; Bowater and Gates, 2015). The nucleotide concentration and diversity in foods depend on their cell density and origin. Therefore, meat (offal), fish and seeds have a high content of nucleic acids and nucleobases whilst milk, eggs and fruit have relatively lower levels although there exist a hierarchy within each of these classification as to relative nucleotide concentrations (Clifford and Story, 1976; Gil, 2002; Garcia-Gomez et al., 2013; Phan et al., 2018; Won et al., 2019). Rovira et al. (2012), in a Spanish government report, note that the proteins of 'unicellular organisms' have concentrations of nucleic acids that are seven times higher than those in meat (Ingledew, 1999). Thus, yeast extracts are a rich source of nucleotides and have formed the basis of human and agricultural and aquaculture feedstuff additives (Tibbets, 2002; Li et al. 2007; Alizadeh et al., 2016).

Nucleotide concentrations and purine and pyrimidine diversity in foods are poorly characterised (Van Buren et al., 1994; Phan et al., 2018). Few foods have had their nucleotide concentrations directly measured with most concentrations in foods having been estimated (Clifford and Gil, 1976; Gil, 2002; Ting et al., 2021). Several recent methodological papers

that have considered the measurement of nucleotides/nucleobases in foods have noted decrements in nucleotide concentration with declining freshness/storage (Zhao et al., 2018; Won et al., 2019). Such observations therefore raise consideration of not only the initial concentration of nucleotides found in foods but also how preservation approaches may alter the nucleotide bioavailability expressed in these foods (Karim et al., 2019; Pott et al., 2020). To this end in both animal and humans the provision of concentrated nucleotide supplements as either a stand-alone additive or incorporated into food have been developed and shown to be important for health (Van Buren et al., 1994; Carver, 2003; Li et al., 2007; Verkerk and Gil, 2009; Jang and Kim, 2019). This exogenous supplementation of dietary nucleotide has been shown to impact upon the growth and development of cells, particularly those that have a rapid turnover and include cellular components of the immune system and the enterocytes of gastrointestinal tract in animal and clinical human models.

Under a normal omnivorous diet in ‘healthy adults’, exogenous nucleotide supplementation is generally not required since the endogenous *de novo* and salvage pathways may be adequate to independently meet daily purine and pyrimidine nucleotide turnover requirements (Kulkarni et al., 1994; Van Buren et al., 1994; Jyonouchi, 1994; Scopesi et al., 2006; Hess and Greenberg, 2012; Lane and Fan 2015). Similarly, Alizadeh et al. (2016) and De Souza et al. (2021) observed that yeast-derived-nucleotide supplementation to broiler chickens under low stress housing conditions elicited no effects on immune function or growth and development. Further low stress animal models suggest similar outcomes across the spectrum of the biological effects attributed to increased nucleotide availability (Pelícia et al., 2010; Perricone et al., 2020). However, exceptions exist; in individuals where nutrition is poor or classed as sub-optimal, i.e., dietary sources of nucleotides (offal, liver, kidneys, eggs, shellfish, yeast extracts) are omitted from the diet and/or where a clinical/physiological trauma, pathophysiology, rapid growth, repair or physical stress is expressed. Such factors likely elevate nucleotide demand above the capacity for endogenous *de-novo* biosynthesis and salvage pathways to meet these imposed demands there may be a case for exogenous (dietary) incorporation/supplementation to facilitate nucleotide augmentation of these pathways (Grimble and Westwood, 2001; Maldonado, et al., 2001; Sancho-Pozo and Gil 2002).

In humans where growth and development is rapid i.e. pre-term infants/infants and diet is deficient i.e., bottle feeding/cow’s milk nucleotide incorporation into formula and food

designated for infants and mothers is recommended and considered conditionally essential (Merolla et al., 2000; Yu, 2002; Carver, 2003; Singhall et al., 2010; Wang et al., 2015; Upadhaya and Kim, 2021). Such observations are also strongly supported in porcine, rodent, ruminants, crustaceans, fish and poultry models which demonstrate efficacy of nucleotides incorporation into feedstuffs and similarly adverse effects are noted when omitted across a range of biological systems and functions (Lin et al., 2009; Chiofalo et al., 2011; Jung and Batal, 2012; Trevesi et al., 2017; Guo et al., 2019; Correa et al., 2021; Goppi et al., 2020; Mohamed et al., 2020; Tan et al., 2021). Recommended daily allowances (RDA) for purine and pyrimidine nucleotide intake through diet or nutritional supplements in adult humans have not been officially set although the upper limit of ingestion which would indicate what is termed the ‘no adverse effect level’ is designated by the European Commission 96/4/EC as 2000 mg·day or 33 kg·bw⁻¹ for purines alone and may be several times higher for pyrimidines based on estimation (Waslien et al., 1970; Verkerk and Gil, 2009; Rovira et al., 2012). The rationale for the limits on purine intake relates to its excretion pathway whereby purines are converted to uric acid and elevate the associated risk of gout, vascular inflammation, metabolic dysregulation, and type 2 diabetes that develop from chronically elevated uric acid levels (Degehan et al., 2008; Zhang et al., 2022). An upper limit of between 33-67 kg·bw⁻¹ (~2-4.6g) of purines for 5-10 days may be required to elicit acute elevations in serum uric acid concentrations above clinical thresholds (Verkerk and Gil, 2009; Coelho et al., 2020). It is presently unknown what effect a mix of purine and pyrimidine nucleotides chronically supplemented in healthy adults may have on uric acid levels.

To bring about physiological adaptation, the general overload model indicates that a period of stress sufficient to disrupt homeostasis in physiological systems followed by a period of recovery will facilitate physiological compensation and improvements in performance (Fry et al., 1992). This cyclical process of exercise stress and recovery forms the basis of effective exercise training prescription to improve endurance, high intensity, and resistance related exercise performance (Glaister et al., 2005; Spencer et al., 2005; Cheng et al., 2020; Parmar et al., 2021). However, if the stress is incorrectly applied and/or not balanced with adequate recovery, this positive adaptative stress can evolve along pathway to maladaptation, leading to emergence of functional impairments in multiple physiological systems often referred to as overreaching/overtraining syndrome (Meeusen et al., 2013; Cadegiani et al., 2017; Grandou et al., 2019; Cheng et al., 2021).

In exercise science literature, the stress-overload model and its interactions with physiological, dietary, psychological, and environmental factors have been shown to contribute to both acute and chronic impairments and dysfunctions in gastrointestinal morphology and function (Dokladny et al., 2016; Costa et al., 2017). These factors also impact innate and adaptive immune function (Walsh et al., 2011), increase susceptibility to illness (Nieman and Wentz, 2019), and impair muscle energetics, repair, and growth (Cheng et al., 2020; Uberti et al., 2020). Given the demonstrated effectiveness of exogenous nucleotide supplementation in supporting these systems in animal models, as well as in malnourished children and adults (Vasquez-Garibay et al., 2006), it is warranted to determine whether similar benefits of exogenous nucleotides might be observed in athletic or exercise contexts, particularly in high-intensity exercise models.

Although most athletes' diets and their use of dietary supplements satisfy macro- and micronutrient requirements, the intake and balance of purine and pyrimidine nucleotides in their diets remain undetermined. Most supplemental protein and whey isolates contain low concentrations of purines with slightly higher levels of pyrimidine's nucleotides present in whey (Gil and Sanchez-Medina, 1981; Schlimme et al., 2000; Mateo, 2005). Consequently, a nucleotide deficit may exist in the athletic diet relative to the physiological stress imposed by training (Koeppel, 2011). Under conditions of high physiological stress, exogenous nucleotide supplementation to augment the body's own supply may shift from being semi-essential to essential (Carver, 1999; Sanchez-Pozo and Gil, 2002; Koeppel et al., 2011). This is important given that purine and pyrimidine nucleotide degradation and loss increase significantly during high-intensity exercise, particularly when performed repeatedly over a short time interval (Hellsten et al., 1998; Hellsten et al., 1999; Stathis et al., 2005; Ipata et al., 2011, Dudzinska, et al., 2010).

Short sprints (<10 sec), with brief recovery intervals (<60 sec), consisting of complete rest or low- to moderate-intensity activity, performed over extended periods of time are a characteristic of movement patterns seen team sports. This activity pattern, termed repeated sprint ability (RSA), is considered essential for intermittent team sports (Buchheit et al., 2011; Girard et al., 2011). RSA is considered an important fitness requirement in intermittent team sports (Bishop et al., 2011; Clemente et al., 2021). Despite this view that supplemental nucleotides may be beneficial in athletes, the optimal dosage and duration of supplementation required to produce effective ergogenic responses remain unclear. Additionally, no data

currently exist regarding the relative balance of purine and pyrimidine nucleotides in each supplement administered in the literature, which may influence exercise outcomes (McNaughton et al., 2007; Ostojic et al., 2012; Ostojic et al., 2013; Riera et al 2013; Casajus et al., 2014; Sterczala et al., 2016).

Studies on nucleotide dosing in the literature report daily intake levels of dietary nucleotides per day of 50 mg (Ostojic et al., 2013), 480 mg (McNaughton et al., 2007), 480 mg (Riera et al 2013), 600mg (Casajus et al., 2014), 278 mg (Sterczala et al., 2016) over periods from 14 days (Ostojic et al., 2013; Sterczala et al., 2016) to 60 days (McNaughton et al., 2007). Variations in dosing may reflect differences in nucleotide delivery methods i.e., oral versus sublingual and the impact on potential bioavailability (McNaughton et al., 2007; Ostojic et al., 2012; Ostojic et al., 2013). The bioavailability of orally administered nucleotides may be low due to potential degradation in the gastrointestinal tract by intestinal nucleotidases, absorption by nucleoside transporters in the intestinal membrane, retention in the enterocytes, and increased hepatic first-pass metabolism (Carver, 1999; Gil, 2002; Hess and Greenberg, 2012; Ting et al., 2021). Consequently, bioavailability of orally delivered nucleotides may be less than 10% of initial dosing level after 28 days supplementation (Coolen et al., 2011) although others have disputed this (Rapaport et al., 2015; Purpera et al., 2017). Sublingual delivery approaches or those delivered parenterally may avoid these gastrointestinal barriers but may not avoid those imposed at the hepatic level (Gasser et al., 1981; Gimble et al., 2000; Gimble and Westwood 2001). It is also important to observe that in animal studies, where nucleotide efficacy is more pronounced, typically use higher dosing levels; several hundred mg of nucleotide per $\text{kg} \cdot \text{BW}^{-1}$ are the norm (Li et al., 2019; Ridwanudin, et al., 2019). A clear understanding of potential dose response relationships for nucleotide delivery approach, concentrations and supplementation duration is thus essential for evaluating potential ergogenic or adaptogenic effects.

Research on the relationship between high intensity and endurance exercise performance and nucleotide supplementation is limited. Potential endurance performance improvements from exogenous nucleotide supplementation may arise from increased availability in purine and pyrimidine nucleotide pools, enhancing energy availability in muscles or altering mitochondrial oxidative capacity (Perez et al., 2004; Xu et al., 2017). High intensity exercise can lead to the degradation and loss of purine and pyrimidine nucleotides from muscle nucleotide pools, thereby reducing their availability as energy sources. Some studies have

reported improved time to exhaustion in high intensity running after 14 days of sublingual nucleotide supplementation (Ostojic et al., 2012; Ostojic et al., 2013). For example, Sterczala et al. (2016) found enhanced muscle force recovery and reduced force loss in nucleotide supplemented resistance trained athletes compared to a placebo undertaking one repetition max assessments. Similarly, Xu et al. (2017) reported a significant extension in swim performance time to exhaustion in a rodent tail weighted forced swimming model with nucleotide supplementation relative to placebo. However, other studies have observed no ergogenic effects from nucleotide supplementation following cycle ergometer Wingate sprints (Mc Naughton et al., 2006; Mc Naughton et al., 2007) or exercise to exhaustion during an incremental graded ramp test (Riera et al., 2013; Casajus et al., 2014). While single purine nucleotide supplements such as ATP have shown potential ergogenic effects at doses exceeding 400mg. (Jordan et al., 2004; Wilson et al., 2013; Rathmacher et al., 2012; Jager et al., 2021), these findings are inconsistent (Herda et al., 2008). The effectiveness of nucleotide supplementation as an ergogenic aid remains inconclusive, with limited data supporting its efficacy. No data exists examining the effects of nucleotides on repeated sprint high intensity exercise.

Nucleotide supplementation has been shown to enhance immune function, improve disease resistance, and reduce mortality in animal and human models under high physiological or cellular stress (Van Buren et al., 1994; Gil 2002; Carver, 2003; Li and Gatlin et al., 2006; Li et al., 2007; Verkerk and Gil, 2009; Hess and Greenberg, 2012; Jang and Kim, 2019). In an athletic context, exercise modulates immune function in an intensity, and duration, dependent manner, with moderate exercise stimulating immunity and sustained high-intensity exercise impairing various in vivo and in vitro cellular and immune humoral immune parameters (Wash et al., 2011; Nieman and Wentz, 2019). Although some studies have observed transient improvements in mucosal immunity and/or attenuation in cortisol release with nucleotide supplementation following high intensity exercise (McNaughton et al., 2006, McNaughton et al., 2007), or submaximal exercise to exhaustion (Riera et al., 2013) others have reported no effect on immune parameters such as secretory IgA secretion or innate and adaptative immune cell counts after exercise (Riera et al., 2013; Ostojic et al., 2013; Sterczala et al., 2016). However, functional improvements in vitro on adaptive immune biomarkers such as lymphocyte proliferation capacity and natural killer cell cytotoxic activity have been reported following exercise in some (Ostojic et al., 2013; Casajus et al., 2014) but not in all cases (Riera et al. 2013). These mixed results suggest that dietary nucleotide supplementation

may have a potential adaptogenic role in immune function, and attenuation of hypothalamic adrenal pituitary axis activation but further research is needed to clarify these effects.

The gastrointestinal (GI) barrier is essential for maintaining the body's internal environment, allowing the selective absorption of nutrients and water while blocking harmful agents by forming a physical barrier, promoting immune protection, and acting as a microbionics host (Farhadi et al., 2003; Bischoff et al., 2010; Sauer et al., 2010; Kinross et al., 2011). The enterocytes of the gastrointestinal system which form the epithelial layers of the mucosa are rapidly dividing tissue, with a rapid turnover rate (whole gut every 72 hours) that have limited capacity for *de novo* synthesis and are functionally dependent upon nucleotide salvage pathways to maintain gastrointestinal barrier proliferation and growth ensuring gut morphology and function (Walker, 2006; Hess and Greenberg, 2012, Ting et al., 2021). Studies in animal and human models have shown that nucleotide supplementation supports GI barrier integrity both morphologically and functionally when 'stress' levels are high (Gil, 2001; Boza et al., 2002; Carver, 2003; María Vieites et al., 2008; Sauer et al., 2012; Che et al., 2016; Valini et al., 2021). They mediate increases in mucin secretion, mucus barrier thickness, increased enterocyte thickness, increased villus crypt depth, increased tight junction protein and antioxidant pathway expression (Meng et al. 2017; Jang and Kim, 2018; Maggiolino et al. 2023). Exercise stress together with its associated thermal stress, blood flow redistribution, and local enterocyte tissue hypoxia may compromise GI barrier function, facilitating the translocation of antigens across the gastrointestinal barrier leading to morphological damage and increased oxidative stress, inflammation, and immune activation (Pals et al., 1997; Dokladny et al., 2016; Costa et al., 2017). Given the positive effects of nucleotide supplementation upon gastrointestinal function noted in animal and human studies it is unknown whether nucleotide supplementation could mitigate GI damage observed following high-intensity exercise.

Literature indicates that exogenous nucleotide supplementation facilitates cell proliferation and growth across various systems, including stem cells (Carver et al., 2004), enterocytes (Sauer et al., 2012), hepatocytes (Perez et al., 2004), lymphocytes (Salobir et al., 2005) and skeletal muscle (Uberti et al., 2020). Animal models including avian (Wang et al., 2021), bovine (Dehghan-Banadaky et al., 2013), porcine (Davila-Ramirez et al., 2020), and aquaculture models (Andriamita et al., 2020) report the addition of nucleotides to feedstuffs increases tissue repair, growth, (Yin et al., 2015) and muscle protein content "Carcass yield"

(Rahmawati et al., 2021; Ciurescu et al., 2021). Similarly, other reports observed that nucleotide supplementation to malnourished human infants increased growth during the early stages of development (Cosgrove et al., 1996; Singhal et al., 2010; Wang et al., 2019). In vitro muscle tissue culture in C₂C₁₂ mouse myotubes observed increased amounts of cell proliferation, differentiation, and growth under non-stress conditions where nucleotides are added to the growth media (Uberty et al., 2020).

Numerous animal studies report that nucleotides exhibit antioxidant and anti-inflammatory effects, which they mechanistically link to explain the effects of nucleotides across physiological systems particularly in promoting cell proliferation, differentiation, and growth (Salobir et al., 2005; Xu et al. 2017; Ti et al., 2019; Tie et al., 2021; Zhu et al., 2021). Specifically, nucleotide supplementation has been associated with increases in antioxidant activity and, elevated anti-inflammatory cytokines expression and the attenuation of pro-inflammatory cytokines in, gut immune and skeletal muscle; all of which are altered during exercise stress.

Following exercise activity, mechanical and oxidative stress levels rise in proportion to the intensity, duration and modality of the activity undertaken, triggering transient systemic inflammation and oxidative stress. This post-exercise response can impair performance and reduce skeletal muscle function, especially when the exercise is novel or repeated without adequate recovery periods (Cheng et al., 2021). While some inflammation is essential for muscle adaptation, repair, and growth, sustained oxidative stress and inflammation, particularly with limited recovery time between sessions, can lead to skeletal muscle dysfunction, impairing muscle performance, repair, and growth (McGee and Hargraves, 2020). Repeated exercise stress has been shown to create an intramuscular environment of chronic inflammation, characterised by elevated immune cell activity and increased release of inflammatory cytokines (e.g., TNF α , IFN γ , and IL-6) (Chen et al., 2020; McGee and Hargraves, 2020). This inflammation is further exacerbated by reactive oxygen species (ROS) production from mitochondria, especially during high-intensity, metabolically demanding exercise. Although nucleotide supplementation has shown potential in promoting cellular growth and repair in animal models (as noted above), no studies to date have specifically investigated nucleotide effects on cell proliferation, differentiation, and growth within a model of skeletal muscle inflammation that simulates exercise-induced stress (Girven et al., 2016). The case as to whether there may exist an adaptogenic role for

nucleotides in this scenario has not been examined.

In conclusion evidence from animal models and to a lesser extent human studies, suggest that exogenous nucleotides supplementation may enhance the structure, enumeration, and function of several physiological systems, especially when subject to either acute and/or chronic stress. However, in human exercise models, the efficacy of exogenous nucleotide supplementation remains unclear, particularly in a high intensity exercise model. The optimal dose and duration of nucleotide supplementation to achieve potential ergogenic and adaptogenic effects across systems, such as gastrointestinal function, immune function, cell metabolism, muscle cell proliferation and growth, are yet to be determined.

1.2 Overall aim of thesis:

The primary aim of this thesis is to examine ergogenic and adaptogenic effects of dietary nucleotides supplementation upon high intensity exercise performance, gastrointestinal permeability, in vivo cell mediated immunity following intermittent high intensity exercise performance. In addition, skeletal muscle growth and proliferation responses to nucleotide supplementation under a high stress cytokine model of tissue inflammation will also be examined.

To achieve the overall aim of this research, the following objectives will be addressed:

1.2.1 Study 1: (Chapter 2)

- To determine the dose response relationships of oral nucleotide supplementation at low, moderate, and high dose concentrations on repeated sprint performance (RSA), blood metabolites and hypothalamic pituitary adrenal axis activation.

1.2.2 Study 2: (Chapter 3)

- To determine the effect chronic oral nucleotide supplementation at a high dose upon gastrointestinal permeability, damage, and symptoms blood metabolites and hypothalamic pituitary adrenal axis (HPA) activation following high intensity repeated sprinting exercise.

1.2.3 Study 3: (Chapter 4)

- To determine at a high nucleotide dose in vivo cell mediated immune responses using a skin contact hypersensitivity model and hypothalamic pituitary adrenal axis (HPA) activation following high intensity repeated sprinting exercise.

1.2.4 Study 4: (Chapter 5)

- To investigate the effect of nucleotide supplementation on murine C₂ C₁₂ myoblast cells and the impact on growth and differentiation in vitro in the absence or presence of inflammation induced via Tumour Necrosis Factor alpha (TNF- α) administration as an exercise mimetic.

Chapter 2

Effect of Oral Nucleotide Supplementation on High Intensity Repeated Sprint Performance (Study 1)

This chapter examines the effects of oral nucleotide supplementation at differing nucleotide doses and durations upon high intensity repeated sprint performance. It presents data from several short-term pilot studies in addition to the primary repeated sprint performance interventions drawn together from the chapters throughout the thesis.

2.1 Abstract

Repeated sprint ability (RSA) is characterised by short-duration high-intensity exercise bouts interspersed with brief recovery periods. During RSA, a proportion of the purine and pyrimidine nucleotide pool is rapidly degraded and exits the muscle potentially compromising performance. Nucleotide loss may be offset either via the purine salvage or via de novo synthesis pathways both of which are slow and metabolically costly. Exogenous nucleotide supplementation has been reported to improve exercise performance after sub-lingual delivery. No data exists on the effect of oral dietary nucleotides upon RSA performance. This chapter investigated the effects of 30-days oral nucleotide supplementation at three dose levels on RSA performance. Forty-three active male participants (age 22.7 ± 4.0 years; ($\dot{V}O_2$ max 49.2 ± 6.4 mL $\cdot$ kg $^{-1}\cdot$ min $^{-1}$) were assigned to one of 4 groups using a double-blind, cross-sectional minimisation of difference design: Placebo ([PL] n= 11), Low dose ([LD] 840 mg $\cdot$ day, n=8), Moderate dose ([MD] 1680 mg $\cdot$ day, n=12) and High dose ([HD]2520 mg $\cdot$ day, n=12). All participants completed a $\dot{V}O_2$ max, RSA familiarisation, and RSA assessment at baseline and post 30 days supplementation. The RSA protocol consisted of 4 sets of 6 x 35 m sprints (15 s active recovery between sprints) with a 5 min inter-set recovery. Each sprint was automatically timed (Brower Timing, USA), HR (Polar s610, Finland) and blood lactate was determined via rapid assay (Arkray, Japan). Data acquired included Sprint Times(s), Fatigue Index, Heart Rate (HR b $\cdot$ min $^{-1}$), and Blood Lactate (mmol $\cdot$ L $^{-1}$). Statistical analysis was undertaken with a four-way mixed ANOVA (SPSS V.21, USA). Data showed no significant main effect or interaction pre- to post-nucleotide supplementation on all variables. Sprint performance: PL 6.1 ± 0.5 vs 6.1 ± 0.6 s; LD 5.9 ± 0.5 , vs 5.9 ± 0.6 s; MD 6.2 ± 0.6 vs 6.1 ± 0.7 s; HD 6.1 ± 0.6 vs 6.1 ± 0.5 s ($P \geq 0.05$). Fatigue Index: PL $14.0 \pm 7.0\%$ vs $14.9 \pm 8.9\%$; LD $14.6 \pm 5.4\%$ vs $13.8 \pm 6.5\%$; MD $12.1 \pm 6.3\%$ vs $12.4 \pm 8.5\%$; HD $12.7 \pm 7.5\%$ vs $14.7 \pm 7.5\%$ ($P \geq 0.05$). Lactate (mmol $\cdot$ L $^{-1}$): PL 10.9 ± 1.9 vs 11.4 ± 2.5 ; LD 11.9 ± 2.0 , vs 11.0 ± 2.5 ; MD 11.4 ± 2.1 vs 13.2 ± 3.0 ; HD 11.6 ± 2.5 vs 13.0 ± 2.2 ($P \geq 0.05$). HR (b $\cdot$ min $^{-1}$): PL 172 ± 11 vs 170 ± 12 ; LD 167 ± 10.0 , vs 167 ± 9.0 ; MD 168 ± 11.0 vs 167 ± 13.0 ; HD 167 ± 12 vs 170 ± 11 ($P \geq 0.05$). Data suggests exogenous nucleotide supplementation at varying administration timings and dose concentrations do not improve high intensity RSA performance, reduce fatigue, or attenuate physiological or blood lactate responses. The result must be considered relative to nucleotide bioavailability levels achieved with oral delivery.

2.2 Introduction

The onset of muscular contractions, whether in isolated muscle fibers or during whole-body muscular activity depends upon the presence of the purine nucleotide adenosine triphosphate (ATP) to facilitate actin-myosin interaction (Frontera and Ochala, 2015). However, ATP supply is limited and must be continually resynthesised to adequately sustain muscle activity. This resynthesis is achieved via a series of interrelated bioenergetic pathways that regenerate ATP from its breakdown products, via a complex regulatory system ensuring adequate energy provision for ongoing muscle contractions (Baker et al., 2010; Ipata, 2011). Typically, cellular ATP concentration is maintained between 1 to 10 mmol·L⁻¹, with a normal ratio of ATP/ADP of approximately 1000 (Zimmermann et al., 2011). Maintaining normal biological function at rest requires an estimated 100 to 150 mmol·L⁻¹ of ATP daily, which means that each molecule of ATP is recycled some 1000 to 1500 times per day (Törnroth-Horsefield and Neutze, 2008). On average, the human body resynthesises an amount of ATP roughly equivalent to its body mass (~65 kg) over a 24-hour period (Rich, 2003). Consequently, during physical activity and exercise, this resynthesis rate increases significantly to meet elevated energy demands (Spencer et al., 2005; Baker et al., 2013).

Adenosine triphosphate is rapidly used during high intensity exercise, particularly in situations involving repeated sprints, where ATP depletion in contracting muscle can reach approximately 20-41% of initial levels (Bogdanis et al., 1994; Hellsten et al., 1998; Hellsten et al., 1999; Stathis et al., 2005; Dudzinska, et al., 2010; Ipata et al., 2011; Egan and Zierath, 2013). Unlike single high intensity efforts, repeated sprints activity (RSA) defined as short bouts of activity (<10 sec), with brief recovery intervals (<60 sec), that may involve complete rest or low- to moderate-intensity activity, places successive demands on muscle metabolic pathways, imposes sequential demands on muscle metabolic pathways. This demand often exceeds the muscle's capacity to rephosphorylate ATP from adenosine diphosphate (ADP), adenosine monophosphate (AMP), and inosine monophosphate (IMP). Each successive exercise bout progressively depletes the total adenine nucleotide (TAN) pool, degrades pyrimidine nucleotides, and reduces the total amount of ATP available in the muscle. This depletion occurs through ATP degradation to inosine monophosphate (IMP), inosine and hypoxanthine, which can exit the muscle and accumulate in the plasma reducing purine availability across the TAN pool (Janssen al., 1987; Tullson et al., 1995; Brault, and Terjung, 2001; Stathis et al., 2005; Bucheit et al., 2011; Girard et al., 2011; Schraner et al., 2020).

Consequently, de novo synthesis and salvage pathways must upregulate their activity to facilitate the restoration and maintenance of the TAN and pyrimidine pool(s) following high intensity exercise (Sheehan, and Tully, 1983; Hellsten-Westing et al., 1993; Yamamoto et al., 2011). However, this upregulation is extremely energy intensive, requiring substantial ATP contribution to support the resynthesis process from IMP combined with de novo nucleotide biosynthesis and salvage recovery (Gil 2002; Garrett, and Grisham, 2016).

While the TAN pool degradation response to high intensity exercise is well characterised, pyrimidine nucleotide degradation and loss has been less frequently characterised (Yamamoto et al., 1997; Dudzinska et al., 2010; Alberghina et al., 2011; Dudzinska et al., 2018) and has primarily been examined in short-term continuous incremental exercise to exhaustion in humans (Dudzinska, et al., 2010; Dudzinska et al., 2018) and equine (Alberghina et al., 2011) models. Presently, data on muscle pyrimidine loss in animal or human models are limited, and little is known about pyrimidine nucleotide loss in response to different types of exercise (Yamamoto et al., 1997; Dudzinska et al., 2010; Alberghina et al., 2011). Yamamoto et al. (1997) have shown that pyrimidine metabolism during sub-maximal exercise at 65% of $\dot{V}O_2$ max for 25 min correlates with the rate of purine metabolism, specifically with ATP availability, showing a 1.5-fold increase in pyrimidine degradation products. Similarly, Dudzinska et al. (2010) found that exercise leading to adenine pool depletion is accompanied by pyrimidine nucleotide co-depletion, resulting in elevated levels of pyrimidine degradation products such as uracil, uridine, and β -alanine in plasma and urine.

The degradation and loss of pyrimidine nucleotides may have significant implications, as they play key roles in cell signalling, neurological function, mitochondrial activity, glucose metabolism, and glycogen synthesis during post-exercise recovery (Ivy, 1991; Dudzinska et al., 2010; Alberghina et al., 2011; Yamamoto et al., 2011). These effects are of importance when intense exercise is performed repeatedly (Bishop et al., 2011; Clemente et al., 2021). Repeated sprint ability (RSA) is considered an important performance factor in intermittent team sports where bursts of short intense exercise (<10s) occur against an aerobic background. Over an exercise period, accumulated loss of both purine and pyrimidine nucleotides may impair performance and slow the rate of recovery (Dudzinska et al., 2010; Alberghina et al., 2011; Dudzinska et al., 2018).

While most athletes' diets and supplements typically meet carbohydrate, protein, and micronutrient requirements, the intake and balance of purine and pyrimidine's nucleotides remains unknown, potentially leading to a nucleotide deficit (Koeppel, 2011; Ostojic et al., 2013). Under such circumstances supplementary nucleotides transition from being semi-essential to become essential dietary components (Sanchez-Pozo and Gil, 2002), particularly given the frequent high-intensity exercise required in training and competition. Exogenous nucleotide supplementation could limit nucleotide degradation, reduce the activation of salvage and de novo biosynthesis pathways, and help maintain total adenine and pyrimidine pools, potentially enhancing mitochondrial function and energy availability in muscle. (Perez et al., 2004; Hess and Greenberg, 2012; Ostojic et al., 2013; Ostojic et al., 2015; Xu et al., 2017). However, research into the effects of exogenous nucleotides as an ergogenic aid remains limited (Mc Naughton et al., 2006; Mc Naughton et al., 2007). Several studies have noted improved time to exhaustion in high intensity running after a 14-day period of sublingual nucleotide supplementation at 50 mg per day (Ostojic et al., 2012; Ostojic et al., 2013). In resistance trained athletes, Sterczala et al. (2016) reported enhanced muscle force recovery and reduced muscle force loss relative to placebo after 14 days of 278 mg nucleotide per day. Xu et al. (2017) observed extended time to exhaustion in a rodent forced swimming model following high dose nucleotide supplementation relative to placebo. Conversely, other studies have observed no ergogenic effects following cycle ergometer Wingate sprints (Mc Naughton et al., 2006; Mc Naughton et al., 2007) or incremental exercise to exhaustion (Riera et al., 2013; Casajus et al., 2014).

While nucleotide supplementation may be potentially beneficial for athletes, the optimal mix of purine and pyrimidine nucleotides, dosage, duration, and delivery method for effective ergogenic outcomes remain unclear (McNaughton et al., 2007; Coolen et al., 2011; Ostojic et al., 2013; Casajus et al., 2014; Sterczala et al., 2016). Supplementing with a single purine nucleotide, ATP, has shown potential ergogenic effects, but only at doses (>400 mg) exceeding those used in these previous studies and inconsistently across literature (Jordan et al., 2004; Rathmacher et al., 2012; Wilson et al., 2013; Purpura et al., 2017; Jäger et al., 2021). Therefore, the effectiveness of purine and pyrimidine nucleotide supplementation as an ergogenic aid remains unproven in human RSA studies. The aim of this study was to determine the effects of oral purine and pyrimidine nucleotide supplementation on RSA performance, as well as associated physiological and metabolic responses, across various dosages and loading protocols.

2.3 Methods

2.3.1 Participants

Participants were recruited from the postgraduate and undergraduate student body of the School for Sport and Exercise Science, Liverpool John Moores University. All participants who volunteered to participate in this study were non-smokers and indicated they were free from acute illness or musculoskeletal injury as ascertained by a readiness to Exercise Questionnaire and health questionnaire to reference potential study exclusion criteria. All participants were trained and recreationally active males and females participating in team sport and endurance activities (Table 2.1.1). Participants were fully informed in writing and followed up with verbal explanation about the nature of the studies, prior to giving their informed consent to participate. Prior to all studies the participants gave verbal confirmation of adherence to pre-test instructions. Participants were asked to confirm adherence to each study's pre-participation requirements. These criteria included abstention from alcohol and exercise in the preceding 24 hours prior to physiological performance. The studies outlined in this chapter were approved by the Research Ethics Committee of Liverpool John Moores University.

Table 2.1.1. Summary of participant's characteristics included within this chapter to examine the possible ergogenic effect of exogenous oral nucleotide supplementation.

<i>Study Designation</i>	<i>N</i>	<i>Gender</i>	<i>Age (years)</i>	<i>Height (cm)</i>	<i>Body Mass (kg)</i>	<i>$\dot{V}O_{2peak}$ (mL·kg⁻¹·min⁻¹) 20-MST</i>
Pilot Study 1	4	4 M	23.0 ± 2.5	182 ± 4.0	79.5 ± 9.4	40.1 ± 2.6
Pilot Study 2	6	4 M	21.8 ± 1.7	182 ± 8.0	77.6 ± 11.4	43.6 ± 6.1
		2 F	23.6 ± 0.7	169 ± 2.8	64.5 ± 6.35	
Pilot Study 3	6	6 M	19.2 ± 1.0	180 ± 6.9	84.4 ± 14.9	45.8 ± 4.7
Chapter 2 (Study 1)	43	43 M	22.7 ± 4.0	175 ± 8.2	72.6 ± 10.8	49.2 ± 6.4
Chapter 3 (Study 2)	15	15 M	21.7 ± 4.2	178 ± 5.0	72.4 ± 6.7	48.9 ± 5.1

2.1.2 Experimental Protocols and Designs

The following section sets out the experimental designs and protocols assigned to each pilot and primary experimental data collection study presented in this chapter.

Table 2.1.2. Summary of experimental design and protocol dosing regimen presented in the pilot studies and main experimental studies 1 and 2 included within this chapter to examine the possible ergogenic effect of exogenous nucleotide supplementation.

<i>Study Designation</i>	<i>Supplement Loading Period (days)</i>	<i>Nucleotide Dosage (mg)</i>	<i>Experimental Design</i>
Pilot Study 1	14-days	840mg	Single-Blind Repeated Measure.
Pilot Study 2	7-days	2520mg	Double-blind, cross over repeated measure. (7-days washout)
Pilot Study 3	7-days	2520mg	Double-blind, cross over measure. (14-days washout period).
Chapter 2 (Study 1)	30-days	Low (840mg) Moderate (1680mg) High (2520mg)	Double-blind, matched pairs design.
Chapter 3 (Study 2)	30-days	High (2520mg)	Double-blind, repeated measure.

Pilot Study 1.

Four recreationally active male participants (Table 2.1.1) volunteered to participate and undertook a single blind cross over repeated measure experimental design (Table 2.1.2). Participants were required to attend the lab on 4 separate visits of varying duration for an initial $\dot{V}O_2$ max fitness assessment via a 20-meter shuttle test (Ramsbottom, 1995) and RAST familiarisation trials. Participants were asked to refrain from exercise for 24 hours prior to physiological assessment. All participants then undertook baseline anthropometry and performance assessment consisting modified repeated anaerobic sprint test consisting of 4 sets of 6 x 35m sprints with a recovery period of 5 minute separating each set. After each sprint, heart rate (HR) (Polar electro, S610i, Kempele, Finland), Ratings of Perceived exertion (RPE) (Borg, 1970; Scale 6-20), and Sprint Times (s) (ST) were recorded. Fatigue index was derived following the procedures of Fitzgerald et al. (1993). A finger prick blood sample (10ul) for Blood Lactate (La) was taken by a safety lancet (Sarstedt, Germany) and analysed by a lactate pro analyser (Lactate Pro, Arkray, Japan) at rest and after each sprint set. Each participant completed a total of 24 sprints (4 sets X 6 sprints) per visit. Upon completion of baseline testing participants were then allocated to a 14-day nucleotide or placebo loading period (n=2), ingesting either 840 mg of nucleotide (840 mg: Nu Cell; Pro-bio, Daresbury, UK) or maltodextrin placebo in anonymised capsules for 14 days. Upon completion of the phase a 7-day washout was undertaken, and participant crossed over to the alternate condition and began the alternate supplementation phase followed by physiological RAST assessment upon completion. Compliance was determined by returned pill count.

Pilot Study 2.

Six recreationally active participants 4 male and 2 female (Table 2.1.1) volunteered to participate and undertook a double-blind cross over repeated measure experimental design (Table 2.1.2). Participants were required to attend the lab on 4 separate visits of varying duration for an initial $\dot{V}O_2$ max fitness assessment via a 20-meter shuttle test (Ramsbottom, 1995) and RAST familiarisation trials. Participants were asked to refrain from exercise for 24 hours prior to physiological assessment. All participants then undertook a baseline anthropometry and performance assessment consisting modified repeated anaerobic sprint test consisting of 4 sets of 6 x 35m sprints with a recovery period of 5 minute separating each set. After each sprint, heart rate (HR) (Polar, Kempele, Finland), Ratings of Perceived exertion (RPE) (Borg, 1970; Scale 6-20), and Sprint Times (s) (ST) were recorded. Fatigue index was

derived following the procedures of Fitzgerald et al. (1993). A finger prick blood sample (10ul) for Blood Lactate (La) was taken by a safety lancet (Sarstedt, Germany) and analysed by a lactate pro analyser (Lactate Pro, Arkray, Japan) at rest and after each sprint set. Each participant completed a total of 24 sprints (4 sets X 6 sprints) per visit. Upon completion of baseline testing participants were then allocated to a 7-day nucleotide or placebo loading period, ingesting either nucleotide (2520mg: Nutri-tide; Pro-bio, Daresbury, UK) derived from yeast (*Saccharomyces cerevisiae*) or a maltodextrin and binding agents' placebo in anonymised capsules. The dosing regimen consisted of 6 capsules 3 times per day after meals. Upon completion of the initial experimental phase a 7-day washout was undertaken, and participants crossed over to the alternate condition and began the alternate supplementation phase followed by physiological RAST assessment upon completion on supplement loading. No supplements were consumed on the day of assessment. Compliance was determined by returned pill count.

Pilot Study 3.

Six recreationally active male participants (Table 2.1.1) volunteered to participate and undertook a double-blind cross over repeated measure experimental design (Table 2.1.2). Participants were required to attend the lab on 4 separate visits of varying duration for an initial $\dot{V}O_2$ max fitness assessment via a 20-meter shuttle test (Ramsbottom, 1995) and RAST familiarisation trials. Participants were asked to refrain from exercise for 24 hours prior to physiological assessment. All participants then undertook a baseline anthropometry and performance assessment consisting modified repeated anaerobic sprint test consisting of 4 sets of 6 x 35m sprints with a recovery period of 5 minute separating each set. After each sprint, heart rate (HR) (Polar, Kempele, Finland), Ratings of Perceived exertion (RPE) (Borg, 1970; Scale 6-20), and Sprint Times (s) (ST) were recorded. Fatigue index was derived following the procedures of Fitzgerald et al. (1993). A finger prick blood sample (10ul) for Blood Lactate (La) was taken by a safety lancet (Sarstedt, Germany) and analysed by a lactate pro analyser (Lactate Pro, Arkray, Japan) at rest and after each sprint set. Each participant completed a total of 24 sprints (4 sets X 6 sprints sets) per visit. Upon completion of baseline testing participants were then allocated to a 7-day nucleotide or placebo loading period, ingesting either nucleotide (2520 mg: Nutri-tide; Probio, Daresbury, UK) derived from yeast (*Saccharomyces cerevisiae*) or a maltodextrin and binding agents' placebo in anonymised capsules. The dosing regimen consisted of 6 capsules 3 times per day after meals. Upon completion of the initial experimental phase a 14-day washout was undertaken, and participants crossed over to the alternate condition and began the alternate supplementation phase followed by physiological RAST assessment

upon completion on supplement loading. No supplements were consumed on the day of assessment. Compliance to the supplementation protocol was determined by returned pill count.

Primary Experimental Investigation 1 (Chapter 2)

Forty-Three recreationally active male participants (Table 2.1.1) volunteered to participate and undertook a double-blind matched pairs design utilising a minimisation of difference protocol to reduce any differences in sprint performance distribution and minimise selection bias across sprint performance (Treasure and Mac Rae, 1998). (Table 2.1.2). Participants were required to attend the lab on 4 separate visits of varying duration for an initial $\dot{V}O_2$ max fitness assessment via a 20-meter shuttle test (Ramsbottom, 1995) and RAST familiarisation trials. Participants were asked to refrain from exercise for 24 hours prior to physiological assessment. All participants then undertook a baseline anthropometry and performance assessment consisting modified repeated anaerobic sprint test consisting of 4 sets of 6 x 35m sprints with a recovery period of 5 minute separating each set. After each sprint, heart rate (HR) (Polar, Kempele, Finland), Ratings of Perceived exertion (RPE) (Borg, 1970; Scale 6-20), and Sprint Times (s) (ST) were recorded. Fatigue index was derived following the procedures of Fitzgerald et al. (1993). A 10 mL venous blood sample was drawn to determine changes in erythrocyte nucleotide concentrations and enzymes. A finger prick blood sample (10ul) for Blood Lactate (La) was taken by a safety lancet (Sarstedt, Germany) and analysed by a lactate pro analyser (Lactate Pro, Arkray, Japan) at rest and after each sprint set. Each participant completed a total of 24 sprints (4 sets X 6 sprints) per visit. Upon completion of baseline testing participants were then allocated to a 30-day nucleotide or placebo loading period, ingesting either nucleotide (Nutri-tide; Pro-bio-UK) derived from yeast (*Saccharomyces cerevisiae*) at three designated loading doses [low: 840mg; moderate: 1680 mg; high: 2520mg] or a maltodextrin and binding agents' placebo in anonymised capsules. The dosing regimen consisted of 6 capsules x 3 times per day after meals. Upon completion of the initial experimental supplementation phase participants returned for assessment followed by physiological RAST assessment. No supplements were consumed on the day of assessment. Compliance was determined by returned pill count.

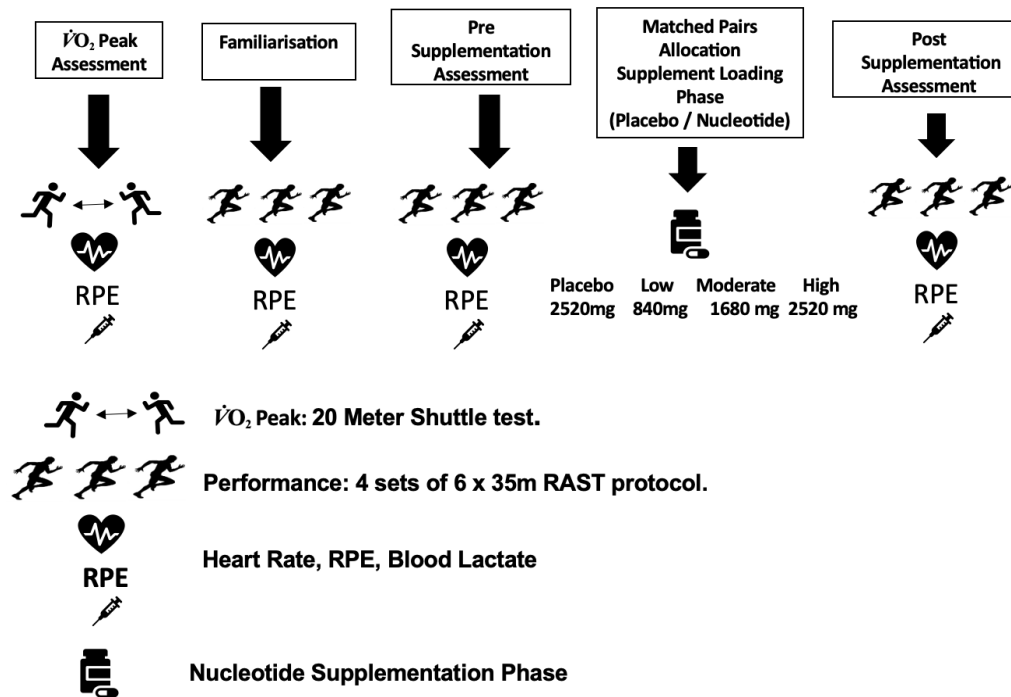


Figure 2.1.1. Experimental design schematic for main experimental study 1 and supplementation exercise trials.

Primary Experimental Investigation 2 (Chapter 3)

Fifteen recreationally active male participants (Table 2.1.1) volunteered to participate and undertook a double-blind repeated measures design utilising to reduce any differences in sprint performance distribution and minimise selection bias across sprint performance (Table 2.1.2). Participants were required to attend the lab on 4 separate visits of varying duration for an initial $\dot{V}O_2$ max fitness assessment via a 20-meter shuttle test (Ramsbottom, 1995) and RAST familiarisation trials. Participants were asked to refrain from exercise for 24 hours prior to physiological assessment. All participants then undertook a baseline anthropometry and performance assessment consisting modified repeated anaerobic sprint test consisting of 4 sets of 6 x 35m sprints with a recovery period of 5 minute separating each set. After each sprint, heart rate (HR) (Polar, Kempele, Finland), Ratings of Perceived exertion (RPE) (Borg, 1970; Scale 6-20), and Sprint Times (s) (ST) were recorded. Fatigue index was derived following the procedures of Fitzgerald et al. (1993). A 10 mL venous blood sample was taken drawn to determine plasma derived biomarkers. A finger prick blood sample (10ul) for Blood Lactate (La) was taken by a safety lancet (Sarstedt, Germany) and analysed by a lactate pro analyser

(Lactate Pro, Arkray, Japan) at rest and after each sprint set. Each participant completed a total of 24 sprints (4 sets X 6 sprints) per visit. Upon completion of baseline testing the participants were then allocated initially to a 30-day nucleotide or placebo loading period (N=7 and 8), ingesting either nucleotide (Nutri-tide; Pro-bio, Daresbury, UK) derived from yeast (*Saccharomyces cerevisiae*) at the designated loading doses of 2520mg or a maltodextrin and binding agents' placebo in anonymised capsules. The dosing regimen consisted of 6 capsules x 3 times per day after meals. Upon completion of the initial experimental supplementation phase participants returned for physiological RAST assessment. A 7 day wash out was undertaken and participant recommenced the experimental design with the alternative treatment condition. No supplements were consumed on the day of assessment. Compliance was determined by returned pill count.

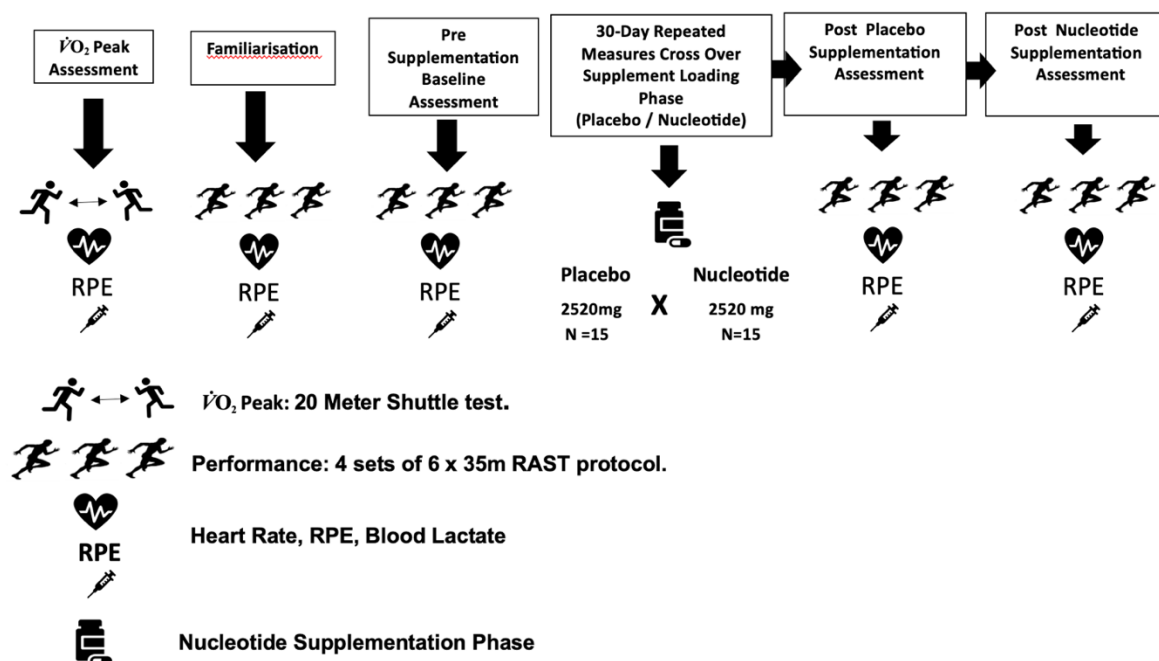


Figure 2.1.2. Experimental design schematic for Main Experimental Study 2 trials.

2.3.3 Anthropometry: Body Mass and Height

Following the provision of informed consent, each participant underwent assessment of anthropometry during the first visit of their respective trials. Using a stadiometer (SECA 213, Cranlea and Co, Bournville, Birmingham, UK), height was measured from the vertex of the cranium to the plantar surface of the foot and recorded in centimetres (cm) to one decimal point. Body mass was also recorded using digital column scales (SECA 704, SECA, UK) and

recorded in kilograms (kg) to one decimal point. The procedures followed the guidelines of the International Society for Kinanthropometry guidelines ISAK (Marfell-Jones et al., 2006).

2.3.4 Cardiorespiratory Measurements: Estimated Maximal oxygen uptake (20-meter shuttle test)

All participants completed an incremental exercise 20-meter multistage shuttle (20-MST) run test on the physiology runway of the TRB building for the estimation of maximal oxygen uptake (Ramsbottom et al., 1985). Prior to beginning the test, a Polar HR monitor was fitted and recorded throughout. The 20-MST running test commenced at $8.5 \text{ km}\cdot\text{h}^{-1}$ and increased by $0.5 \text{ km}\cdot\text{h}^{-1}$ every 1 minute with participants running between two lines set 20 m apart at a pace dictated by an audio signal emitted at appropriate intervals to replicate the running speed required. The test was terminated when the participant failed twice to reach within 3 m of the 20-MST line in time with consecutive tones (objective evaluation) or the participant felt unable to complete another shuttle at the audio signal designated speed (subjective evaluation). The total number of levels and shuttles covered during the 20-MST (including the last incomplete shuttle) was designated as the testing score and converted to an estimated aerobic capacity using a conversion formula (Paradis et al., 2014). Laboratory environmental conditions were recorded during all exercise testing procedures and remained constant with a temperature at $17 \pm 3^\circ\text{C}$ and relative humidity at $48 \pm 5\%$. Rating of perceived exhaustion (RPE) were implemented to anchor the perception of effort to be utilised later during the repeated anaerobic sprint test trials (Draper and Whyte, 1997).

2.3.5 Heart Rate

Heart rate (HR) ($\text{b}\cdot\text{min}^{-1}$) was continually monitored and recorded during all experimental trials by a wearable electrocardiography (Polar FT1, Polar UK). In all studies, heart rate ($\text{b}\cdot\text{min}^{-1}$) was continually measured at 5-second intervals; data as presented represents an average over designated time periods. The polar HR chest strap to determine heart electrical activity was worn directly below the chest at $\sim\text{V5}$ level with the transmitter positioned centrally on the xipho-sternum then adjusted to minimise any movement during each exercise trial. ECG gel was applied to the electrodes to increase conductivity of the signal and improve reliability of measurement in the initial stages of exercise.

2.3.6 Rating of Perceived Exertion (RPE)

RPE was recorded, using the Borg 6-20 (Borg, 1970) scale, at the end of each shuttle stage during the multi-stage maximal oxygen uptake assessment and at the end of each set within the RAST experimental tests. Participants were familiarised using the Borg scale during the initial maximal incremental exercise test to exhaustion. Each end of the Borg scale was ‘anchored’ during the 20-MST maximal exercise protocol so as to introduce the perception of effort associated with progression through the scale from 6 [rest] through 20 [maximal effort], (Birk and Birk, 1987). This process provides a reference point against which participants can refer/gauge their subsequent efforts during exercise trials.

Rating	Description
6	No exertion at all
7	Extremely Light
8	
9	Very Light
10	
11	
12	
13	Somewhat Hard
14	
15	Hard
16	
17	Very Hard
18	
19	Extremely Hard
20	Maximal Exertion

Borg scale for reporting subjective rating of Perceived exertion (Borg, 1970).

2.3.7 Collection, Preparation, Storage and Analysis of Blood Samples

Venous blood samples were collected by a qualified phlebotomist from a superficial vein in the ante-cubital fossa of the forearm using standard venepuncture techniques (Vacutainer Systems, Becton, Dickinson). Briefly, a tourniquet was applied to the upper arm to induce an occlusion in venous return; the medial antecubital vein was cleaned with a Medi-swab isopropyl alcohol wipe BP 70% (Seaton Health Care Group, Oldham, UK). The butterfly needle (BD safety set 21 G $\frac{3}{4}$ Becton Dickinson, Oxford, UK) was inserted, once a flashback was observed, the needle secured, and the tubes were attached. Upon completion of the sampling the needle was removed, and pressure applied to the puncture site to minimise the potential for a subcutaneous haematoma. All blood samples were collected into vacutainers™ of differing volumes (6.0 mL–10 mL) (Becton Dickinson, Northampton UK). The samples were collected into four vacutainers; a serum separation tube, EDTA and lithium heparin which was used as a clotting or chelating agent for the blood. Briefly the serum separation tube was allowed to sit at room temperature for approx. 30 minutes until clotted then placed on ice until centrifugation. The samples were then centrifuged (Sigma 3-18 k Scientific Laboratory Supplies, UK) at 1300 x g for 10 minutes at 4°C (Serum and EDTA) or at 1000 X g for 10 minutes at 4°C. The plasma/serum was then carefully removed from the vacutainer using a pipette (Fisherbrand, Finnpiptette, U.S.A.) and aliquoted into replicates. Following this, plasma and serum were then stored at -80°C (Thermo, ULT Freezer, Ohio U.S.A.).

2.3.8 Capillary Blood Lactate Collection and Analysis

Capillary blood lactate was determined from a finger puncture test. Briefly at the end of each exercise set a finger (1st- 3rd digit) was cleaned with an alcohol swab to remove any sweat or blood and then the skin was penetrated with a sterile single-use lancet (Sarstedt, Germany). Approximately 10 µL of blood was withdrawn via gentle squeezing action upon the finger. Test strips were inserted into a Lactate pro (Arkray, Kyoto, Japan) device which were then placed against the surface of this blood sample until beeps were audible, signifying the start of analysis. The same investigator collected and analysed all blood samples throughout the study. The Arkray Lactate Pro test uses an electrochemical method based on an enzyme reaction to measure blood lactate levels. The assay principle involves the reaction of lactate with lactate oxidase, producing pyruvate and hydrogen peroxide. The resulting hydrogen peroxide is then

detected using a colorimetric probe, and the amount of current generated is directly proportional to the lactate concentration in the sample.

2.3.9 Repeated Anaerobic Sprint Test Exercise Protocol

A high intensity intermittent exercise sprint protocol was performed by each participant. The protocol a modified version of the Draper and Whyte (1997) Repeated Anaerobic Sprint Test (RAST) consisted of 4 sets of 6 x 35 m sprints, with 15 second active run recovery to the start line after each sprint and a 5-minute passive recovery separating each set (Figure 2.1.3). Briefly, the test commenced with the participant undertaking a maximal exertion 35-meter sprint with participant then running back to the start line before commencing the next sprint with a time limit of 15 seconds between each sprint. Each sprint was automatically timed (Brower Timing System, USA), and HR, and RPE (Borg Scale 6-20), were recorded after each sprint. Blood lactate was recorded prior to exercise commencement (baseline) after each set of 6 X 35m sprints and 5 minutes post protocol completion. Fatigue index (FI) was determined using the percentage decrement calculation (Fitzsimmons et al., 1993):

$$\text{Fatigue} = (100 \times [\text{total sprint time} / \text{ideal sprint time}]) - 100$$

Total sprint time= sum of sprint time from all sprints.

Ideal time= number of sprint x fastest sprint time.

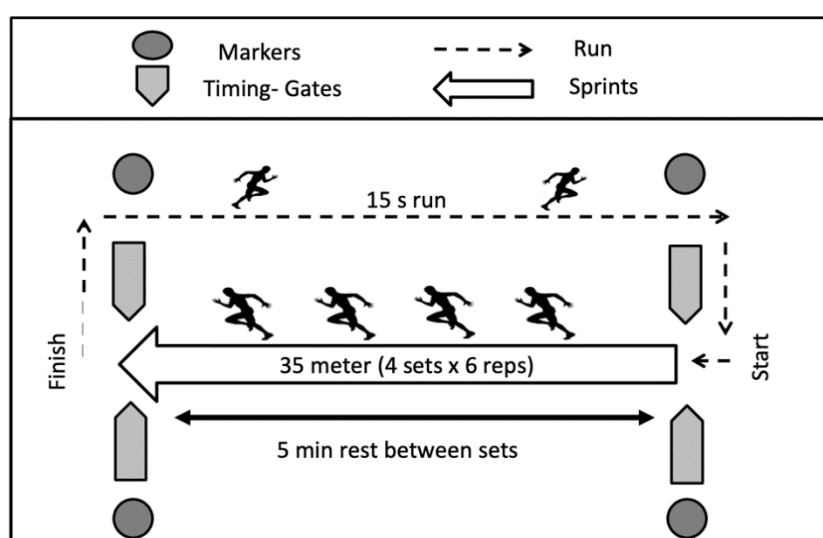


Figure 2.1.3. Protocol schematic for the Repeated Anaerobic Sprint Test (Modified, Draper and Whyte, 1997).

2.3.10 Dietary Nucleotide Supplementation.

Across the studies presented in this chapter participants ingested either a capsule consisting of an outer gelatine shell with either 5-ribonucleotides/RNA nucleotides (Nutri-tide; Pro-bio, Daresbury, UK) derived from yeast (*Saccharomyces cerevisiae*) at the designated loading doses as set out in Table 2.1.2 i.e. 840 mg, 1680 mg, 2520 mg or equivalent maltodextrin and binding agent's placebo in anonymised capsules. The nucleotide formulation was a proprietary mix of purine and pyrimidine nucleotides including the following containing cytosine, uridine, thymidine, adenosine, and guanine. The ratio of each was undisclosed for commercial reasons.

2.3.11 Statistical Analysis

Statistical analysis was carried out using the Statistical package for Social Science software program (SPSS V. 23, for Windows, Chicago, Illinois, USA). All Data was presented as means ($\pm$ SD). In Main Study 1 a two-way mixed and four-way mixed ANOVA (four groups [Placebo, 840 mg, 1680 mg, 2520 mg] sprint performance with pre- post supplementation), within subjects' factor was set as time-point to measure any significant changes in sprint performance, fatigue index, physiological responses (heart rate, RPE), and metabolic (blood lactate). In main study a two mixed design ANOVA was applied to the above parameters. To correct any violations of sphericity the degrees of freedom were corrected using Greenhouse-Geisser epsilon (<0.75) or Huynh-Feldt epsilon (> 0.75), as applicable (Field, 2000). Post-hoc Tukey analysis used Bonferroni correction to adjust multiple comparisons. Statistical significance was set at $p \leq 0.05$ for all tests.

2.4 Results

2.4.1 Pilot Study 1

Data shows no significant effects of nucleotides relative to placebo after 14 days ingestion of 840mg per day upon the variables outlined in Table 2.1.3.

Table 2.1.3. Performance, perceptual and physiological responses to dietary nucleotide supplementation relative to placebo. Data are expressed as Mean $\pm$ SD. Data represent the average taken over the RAST protocol.

Variables (Average 24 sprints)	Placebo (n = 4)	Nucleotide (n = 4)	P value
Sprint Time (s)	7.1 $\pm$ 0.37	6.6 $\pm$ 0.12	$p \geq 0.05$
Fatigue Index (%)	8.43 $\pm$ 2.86	7.66 $\pm$ 2.28	$p \geq 0.05$
Heart Rate (b $\cdot$ min ⁻¹)	178 $\pm$ 3	170 $\pm$ 1	$p \geq 0.05$
RPE (Borg scale)	17 $\pm$ 3	16 $\pm$ 2	$p \geq 0.05$
Blood Lactate (mmol $\cdot$ L ⁻¹)	9.5 $\pm$ 4.71	7.4 $\pm$ 3.53	$p \geq 0.05$

2.4.2 Pilot Study 2

There were no significant effects of nucleotide ingestion upon average sprint time, heart rate, Fatigue Index, RPE and blood lactate following 7 days of 2520 mg per day ingestion upon the variables outlined in Table 2.1.4.

Table 2.1.4. Performance, perceptual and physiological responses to dietary nucleotide supplementation relative to placebo. Data are expressed as Mean $\pm$ SD. Data represents the average taken over the RAST protocol.

Variables (Average 24 sprints)	Placebo (n = 6)	Nucleotide (n = 6)	P value
Sprint Time (s)	5.45 $\pm$ 0.06	5.37 $\pm$ 0.06	$p \geq 0.05$
Fatigue Index (%)	10.43 $\pm$ 3.46	11.66 $\pm$ 2.11	$p \geq 0.05$
Heart Rate (b $\cdot$ min ⁻¹)	153 $\pm$ 8	162 $\pm$ 8	$p \geq 0.05$
RPE (Borg scale)	12 $\pm$ 2	12 $\pm$ 2	$p \geq 0.05$
Blood Lactate (mmol $\cdot$ L ⁻¹)	11.4 $\pm$ 2.94	8.68 $\pm$ 3.87	$p \geq 0.05$

2.4.3 Pilot Study 3

No significant effect in sprint time, fatigue Index, heart rate, RPE and blood lactate after 14 days ingestion of 2520mg per day upon the variables outlined in Table 2.1.5.

Table 2.1.5. Performance, perceptual and physiological responses to dietary nucleotide supplementation relative to placebo. Data are expressed as Mean $\pm$ SD. Data represents the average taken over the 24 sprint RAST protocol.

Variables	Placebo (<i>n</i> = 6)	Nucleotide (<i>n</i> = 6)	<i>P</i> value
Sprint Time (s)	5.16 $\pm$ 0.48	5.15 $\pm$ 0.41	<i>p</i> $\geq$ 0.05
Fatigue Index (%)	6.23 $\pm$ 2.86	5.37 $\pm$ 2.28	<i>p</i> $\geq$ 0.05
Heart Rate (b $\cdot$ min ⁻¹)	175 $\pm$ 6	171 $\pm$ 7	<i>p</i> $\geq$ 0.05
RPE (Borg scale)	13 $\pm$ 1	13 $\pm$ 1	<i>p</i> $\geq$ 0.05
Blood Lactate (mmol $\cdot$ L ⁻¹)	10.6 $\pm$ 2.60	11.5 $\pm$ 2.45	<i>p</i> $\geq$ 0.05

2.4.4 Main Experimental Study 1 (Data presented here relates to Chapter 2)

2.4.4.1 Participants Characteristics

The anthropometric and physical characteristics of the participants in main study 1 Characteristics of participants are presented in Table 2.1.6.

Table 2.1.6. Anthropometric and physiological characteristics of the participants partitioned by experimental group allocation (Mean $\pm$ SD).

Variables	Placebo (<i>n</i> = 11)	Nucleotide Low Dose (<i>n</i> = 8)	Nucleotide Moderate Dose (<i>n</i> = 12)	Nucleotide High Dose (<i>n</i> = 12)
Age (years)	22.3 $\pm$ 5.6	21.9 $\pm$ 1.9	21.3 $\pm$ 2.3	25.0 $\pm$ 4.2
Weight (kg)	75.2 $\pm$ 9.2	77.8 $\pm$ 13.9	68.7 $\pm$ 12.4	70.8 $\pm$ 6.9
Height (cm)	177.1 $\pm$ 8.5	178.0 $\pm$ 9.5	171.0 $\pm$ 5.5	175.8 $\pm$ 8.5
Body Mass Index (kg $\cdot$ m ²)	24.0 $\pm$ 2.4	24.4 $\pm$ 3.1	23.3 $\pm$ 3.4	22.9 $\pm$ 2.0
$\dot{V}O_{2peak}$ (mL $\cdot$ kg ⁻¹ $\cdot$ min ⁻¹)	49.4 $\pm$ 5.5	51.5 $\pm$ 5.2	47.4 $\pm$ 6.67	49.4 $\pm$ 7.6

2.4.4.2 Repeated Anaerobic Sprint Test Performance

All participants successfully completed the 4 sets of 6 x 35m high intensity intermittent exercise sprint protocol. There were no significant main effects or interaction effects of nucleotide supplementation across all doses upon sprint performance ($F_{19,7\ 256.5} = 1.02$, $P = 0.45$). Average sprint times across sets both pre and post supplementation declined significantly with time within each set ($P < 0.05$). Table 2.1.7 further indicates RSA times across conditions and sets. Whilst figure 2.1.4 presents the mean of the 24 sprints.

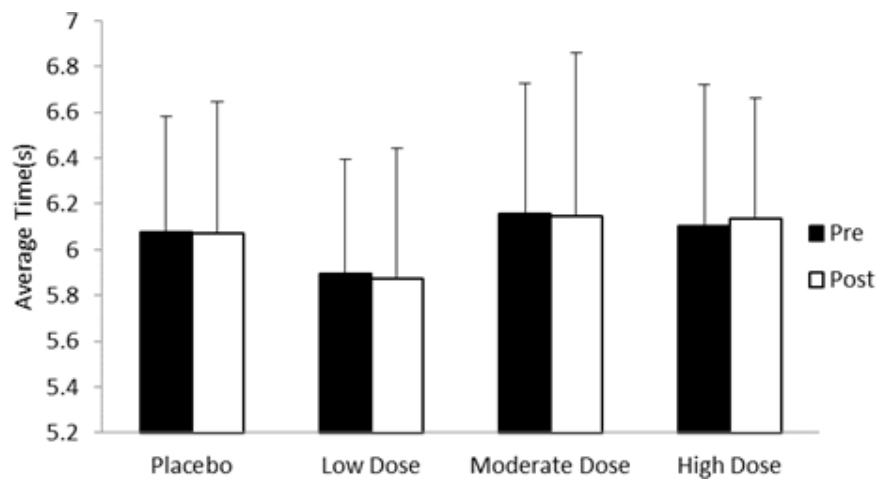


Figure 2.1.4. Mean sprint times (s) across supplementation dosing regimen pre and post supplementation. Data are expressed as Mean $\pm$ SD.

Table 2.1.7. Repeated Anaerobic Sprint Test performance (s) of participants across conditions and sets (4sets x 6 rep). No significant effects of dose/condition pre and post supplementation were evident. Data are expressed as Mean $\pm$ SD.

Group	Pre-Supplementation Time (s) per set.				Post-Supplementation Time (s) per set.				P Value
	1	2	3	4	1	2	3	4	
Placebo	5.78 $\pm$ 0.41	6.04 $\pm$ 0.47	6.21 $\pm$ 0.56	6.27 $\pm$ 0.58	5.87 $\pm$ 0.47	6.09 $\pm$ 0.70	6.18 $\pm$ 0.55	6.14 $\pm$ 0.57	$p \geq 0.05$
Low Dose	5.67 $\pm$ 0.32	5.92 $\pm$ 0.54	5.99 $\pm$ 0.55	6.00 $\pm$ 0.60	5.68 $\pm$ 0.51	5.85 $\pm$ 0.52	5.95 $\pm$ 0.56	6.01 $\pm$ 0.70	$p \geq 0.05$
Mod Dose	6.0 $\pm$ 0.52	6.10 $\pm$ 0.55	6.34 $\pm$ 0.63	6.19 $\pm$ 0.58	5.88 $\pm$ 0.43	6.12 $\pm$ 0.65	6.31 $\pm$ 0.89	6.27 $\pm$ 0.91	$p \geq 0.05$
High Dose	5.81 $\pm$ 0.44	6.0 $\pm$ 0.47	6.30 $\pm$ 0.84	6.31 $\pm$ 0.71	5.88 $\pm$ 0.39	6.12 $\pm$ 0.47	6.21 $\pm$ 0.62	6.33 $\pm$ 0.62	$p \geq 0.05$

Table 2.1.8. Repeated Anaerobic Sprint Test Fatigue Index (%) of participants No significant effects of dose/condition were apparent pre and post supplementation were evident. Data are expressed as Mean $\pm$ SD.

Group	Pre-Supplementation Fatigue Index (%) per set.				Post-Supplementation Fatigue Index (%) per set.				P Value
	1	2	3	4	1	2	3	4	
Placebo	10.9 $\pm$ 6.1	15.1 $\pm$ 7.2	14.4 $\pm$ 7.0	15.4 $\pm$ 7.7	11.2 $\pm$ 7.2	15.9 $\pm$ 10.5	15.3 $\pm$ 8.8	16.9 $\pm$ 9.1	$p \geq 0.05$
Low Dose	11.7 $\pm$ 3.0	13.6 $\pm$ 5.0	16.9 $\pm$ 6.1	16.1 $\pm$ 7.7	10.0 $\pm$ 4.7	14.9 $\pm$ 7.0	15.5 $\pm$ 5.7	14.6 $\pm$ 8.7	$p \geq 0.05$
Mod Dose	11.6 $\pm$ 6.3	12.0 $\pm$ 5.6	12.9 $\pm$ 6.4	11.8 $\pm$ 6.7	10.2 $\pm$ 5.7	13.7 $\pm$ 10.0	13.0 $\pm$ 11.3	12.4 $\pm$ 6.7	$p \geq 0.05$
High Dose	9.5 $\pm$ 5.3	12.3 $\pm$ 6.3	14.1 $\pm$ 9.36	14.8 $\pm$ 9.1	10.3 $\pm$ 5.2	15.8 $\pm$ 7.6	15.5 $\pm$ 8.2	17.0 $\pm$ 8.8	$p \geq 0.05$

2.4.4.3 Fatigue Index Data

There was no significant main effect of nucleotide supplementation at any dose upon the fatigue index (%) during sprint performance or significant interaction effect present ($F_{7.48, 97.2} = 0.18$, $P = 0.99$). Table 2.1.8 presents data of fatigue index (%) across placebo and dosing conditions.

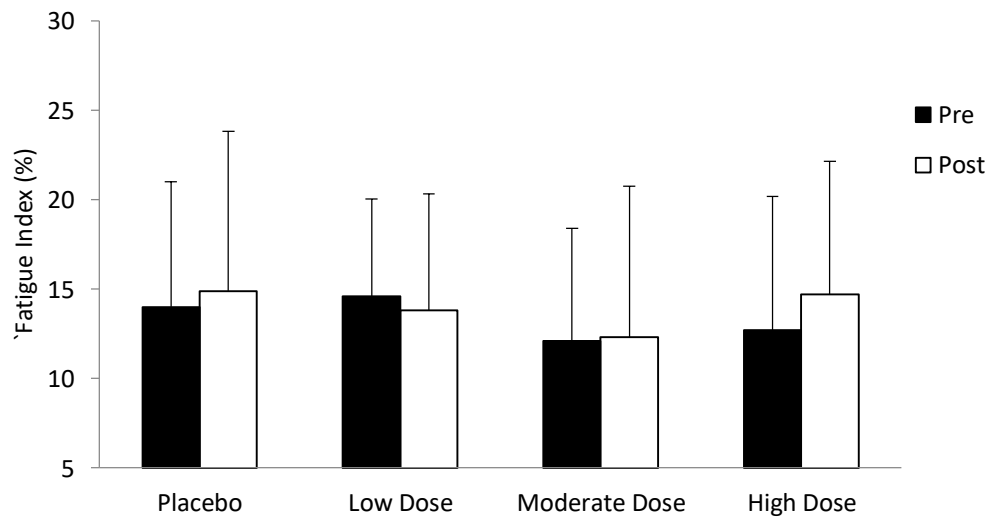


Figure 2.1.5. Mean fatigue index (%) across dosing regimen pre and post supplementation. Data are expressed as Mean $\pm$ SD.

2.4.4.4 Heart Rate Data

During pre- and post-supplementation phase, the mean heart rate for all 4 sprints sets combined, indicated no significant main effect of nucleotide supplementation at any dose upon the heart rate responses during sprint performance or no significant interaction effects ($F_{11.9, 156} = 0.92$, $P = 0.53$). Heart rate increased significantly with exercise sprint performance within each set from rest ($P < 0.05$).

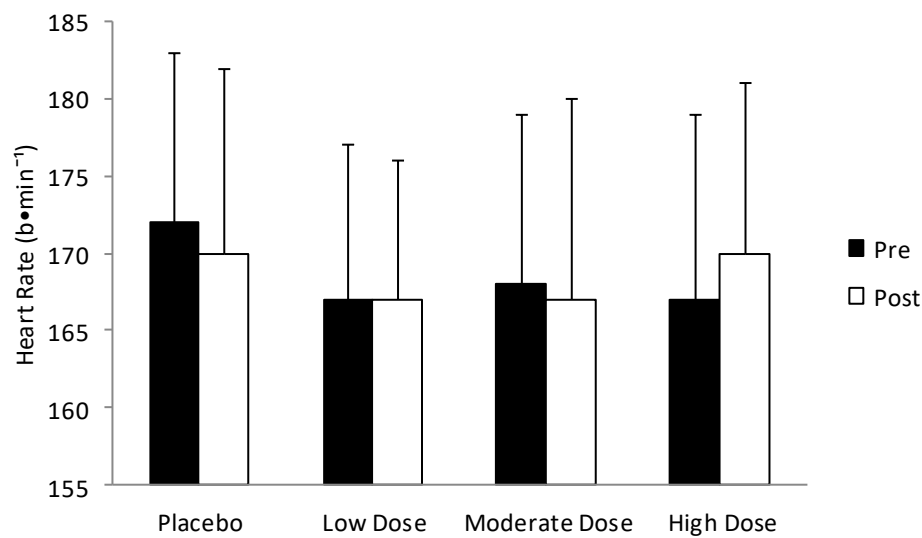


Figure 2.1.6. Mean heart rate (b·min⁻¹) across dosing regimen pre and post supplementation. Data are expressed as Mean $\pm$ SD.

Table 2.1.9. Heart Rate ($\text{b} \cdot \text{min}^{-1}$) responses of participants (Mean $\pm$ SD) ordered by set. No significant effects of dose pre and post supplementation were evident.

Group	Pre-Supplementation Heart Rate per set.				Post-Supplementation Heart Rate per set.				P Value
	1	2	3	4	1	2	3	4	
Placebo	167 $\pm$ 14	173 $\pm$ 10	171 $\pm$ 11	174 $\pm$ 8	166. $\pm$ 14	170 $\pm$ 10	170 $\pm$ 11	172 $\pm$ 13	$p \geq 0.05$
Low Dose	158 $\pm$ 15	168 $\pm$ 9	170 $\pm$ 9	171 $\pm$ 8	161 $\pm$ 11	166 $\pm$ 9	169 $\pm$ 9	170 $\pm$ 9	$p \geq 0.05$
Mod Dose	162 $\pm$ 15	169 $\pm$ 10	169 $\pm$ 8	172 $\pm$ 10	162 $\pm$ 17	168 $\pm$ 12	169 $\pm$ 10	171 $\pm$ 10	$p \geq 0.05$
High Dose	155 $\pm$ 16	168 $\pm$ 12	170 $\pm$ 8	172 $\pm$ 10	167 $\pm$ 14	170 $\pm$ 10	170 $\pm$ 11	173 $\pm$ 10	$p \geq 0.05$

Table 2.2.0. RPE (AU) responses of participants (Mean $\pm$ SD) ordered by set. No significant effects of dose pre and post supplementation were evident.

Group	Pre-Supplementation RPE per set.				Post-Supplementation RPE per set.				P Value
	1	2	3	4	1	2	3	4	
Placebo	13 $\pm$ 3	15 $\pm$ 3	16 $\pm$ 2	16 $\pm$ 2	14 $\pm$ 2	16 $\pm$ 2	16 $\pm$ 2	16. $\pm$ 2	$p \geq 0.05$
Low Dose	14 $\pm$ 2	16 $\pm$ 3	16 $\pm$ 2	17 $\pm$ 2	14 $\pm$ 2	17 $\pm$ 2	17 $\pm$ 2	16 $\pm$ 2	$p \geq 0.05$
Mod Dose	11 $\pm$ 3	12 $\pm$ 3	13 $\pm$ 3	15 $\pm$ 3	12 $\pm$ 3	13 $\pm$ 4	14 $\pm$ 3	17 $\pm$ 3	$p \geq 0.05$
High Dose	13 $\pm$ 2	15 $\pm$ 2	15 $\pm$ 2	16 $\pm$ 2	13 $\pm$ 2	15 $\pm$ 1	16 $\pm$ 1	17 $\pm$ 2	$p \geq 0.05$

Table 2.2.1. Blood lactate responses ($\text{mmol}\cdot\text{L}^{-1}$) responses of participants (Mean $\pm$ SD) ordered by set. No significant effects of dose pre and post supplementation were evident.

Group	Pre-Supplementation Blood Lactate per set.				Post-Supplementation Blood Lactate per set.				P Value
	1	2	3	4	1	2	3	4	
Placebo	9.1 $\pm$ 2.0	11.4 $\pm$ 1.5	11.6 $\pm$ 1.5	11.7 $\pm$ 2.3	9.1 $\pm$ 2.9	11.9 $\pm$ 2.7	11.6 $\pm$ 2.0	12.5 $\pm$ 2.0	$p \geq 0.05$
Low Dose	10.2 $\pm$ 2.0	11.6 $\pm$ 1.4	13.0 $\pm$ 2.3	13.1 $\pm$ 2.2	9.9 $\pm$ 2.1	10.5 $\pm$ 2.5	12.1 $\pm$ 2.0	11.8 $\pm$ 2.0	$p \geq 0.05$
Mod Dose	9.6 $\pm$ 2.6	11.4 $\pm$ 2.1	11.9 $\pm$ 1.9	12.9 $\pm$ 1.5	11.8 $\pm$ 3.0	14.5 $\pm$ 3.4	13.1 $\pm$ 3.3	13.5 $\pm$ 3.3	$p \geq 0.05$
High Dose	9.5 $\pm$ 2.6	11.1 $\pm$ 2.6	13.0 $\pm$ 2.1	13.0 $\pm$ 2.7	11.1 $\pm$ 2.6	13.2 $\pm$ 2.5	14.0 $\pm$ 2.1	13.8 $\pm$ 1.6	$p \geq 0.05$

2.4.4.5 Rating of Perceived Exertion (RPE) Data

During pre- and post-supplementation phase, the mean RPE for all 4-sets combined, indicated no significant effect of nucleotide supplementation at any dose upon the rating of perceived exertion during sprint performance pre and post supplementation ($F_{20.9, 272.4} = 0.88$, $P=0.62$). In addition, there was no significant interaction between all conditions ($P \geq 0.05$). RPE showed a significant effect of time increasing significantly over the duration of the 4 sets ($P \geq 0.05$).

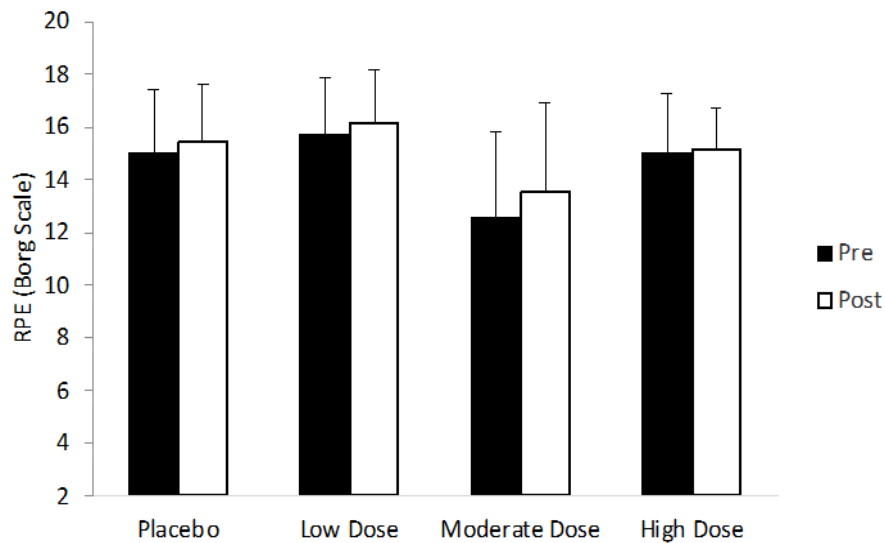


Figure 2.1.7. Mean RPE (Borg scale) across all 24 sprints categorised by dosing regimen pre and post supplementation. Data are expressed as Mean $\pm$ SD.

2.4.4.6 Blood Lactate Data

The mean blood lactate for all 4-set combined, indicated there no significant main effect of nucleotide supplementation at any dose on blood lactate concentrations or significant interaction effects during each set of sprint performance pre and post ($F_{9,117} = 0.66$, $P = 0.74$).

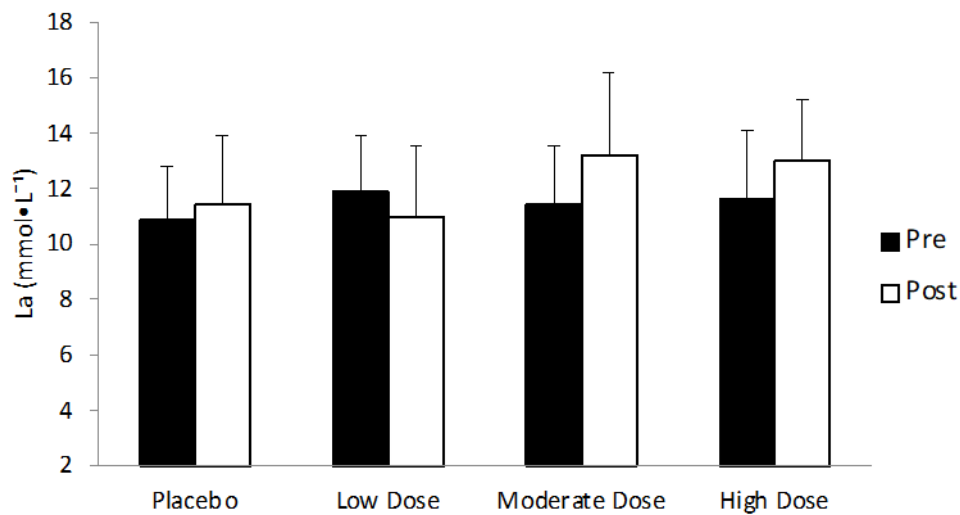


Figure 2.1.8. Mean blood lactate ($\text{mmol}\cdot\text{L}^{-1}$) concentration across all 24 sprints dosing categorised by dosing regimen pre and post supplementation. Data are expressed as Mean $\pm$ SD.

2.4.4.7 Uric Acid Data

The mean uric acid for all 5 time points; pre-supplementation, day-15 during the loading phase, after 30-days of supplementation, post 14-days and 28-days after supplement were no significant main effects of nucleotide supplementation at any dose on uric acid concentrations during pre-test assessment ($F_{10.1, 131.4} = 1.37$, $P = 0.20$).

Table 2.2.2. Resting Plasma Uric acid concentrations at 5 time points prior to, during and post experimental nucleotide and placebo supplementation regimen (Data are expressed as mean $\pm$ SD).

Group	Uric Acid ($\mu\text{mol}\cdot\text{L}^{-1}$)					P value
	Day 0	Day 15	Pre-test Day 30	Post 14-days	Post 28 days	
Placebo	373.5 $\pm$ 80.3	311.6 $\pm$ 56.3	315.2 $\pm$ 109.3	358.4 $\pm$ 54.1	361.0 $\pm$ 49.8	$p \geq 0.05$
Low Dose	325.0 $\pm$ 134.3	359.2 $\pm$ 145.4	315.8 $\pm$ 115.9	323.1 $\pm$ 48.1	326.9 $\pm$ 131.9	$p \geq 0.05$
Mod Dose	346.0 $\pm$ 88.7	362.1 $\pm$ 117.1	325.2 $\pm$ 111.6	334.8 $\pm$ 123.1	331.3 $\pm$ 102.3	$p \geq 0.05$
High Dose	302.9 $\pm$ 127.9	283.5 $\pm$ 85.3	330.3 $\pm$ 93.0	322.2 $\pm$ 84.6	325.1 $\pm$ 75.2	$p \geq 0.05$

2.4.5 Main Experimental Study 2

This section of data presents the ergogenic data responses from chapter 3. The premise for incorporating the data from this study here is to consider all the ergogenic responses in one place rather than distributed across studies.

2.4.5.1 Participants Characteristics

Characteristics of participants who participated are presented in Table 2.2.3.

Table 2.2.3. Participant characteristics of main experimental study 2. Data are expressed as Mean $\pm$ SD.

Variables	Participants ($n = 15$)
Age (years)	21.7 $\pm$ 4.2
Weight (kg)	72.4 $\pm$ 6.7
Height (cm)	177.7 $\pm$ 5.0
$\dot{V}O_2$ Peak (mL $\cdot$ kg ⁻¹ $\cdot$ min ⁻¹)	49.0 $\pm$ 5.1

2.4.5.2 Repeated Anaerobic Sprint Test Performance

Average sprint times across sets pre and post supplementation are presented (Table 2.2.4).

Table 2.2.4 Sprint performance output of different supplement condition. No significant effects of dose pre and post supplementation were evident. Data are expressed as Mean $\pm$ SD.

Supplement Condition	Sprint Time (s)				P value
	Set 1	Set 2	Set 3	Set 4	
Baseline	5.94 $\pm$.054	6.12 $\pm$ 0.46	6.30 $\pm$ 0.50	6.30 $\pm$ 0.52	$p \geq 0.05$
Post Placebo	6.01 $\pm$ 0.44	6.22 $\pm$ 0.56	6.41 $\pm$ 0.59	6.55 $\pm$ 0.70	$p \geq 0.05$
Post Nucleotide	5.93 $\pm$ 0.48	6.07 $\pm$ 0.47	6.30 $\pm$ 0.51	6.46 $\pm$ 0.76	$p \geq 0.05$

During pre-supplementation phase, the mean sprint performance for all 4 sets combined, were 6.17 ± 0.47 s while post 30-days placebo loading were 6.30 ± 0.56 s and the post 30-days nucleotide loading were 6.19 ± 0.52 s (Figure 2.1.9). There were no significant main effects or interaction effects of 30-days nucleotide supplementation upon sprint performance ($F_{3.1, 43.7} = 1.27$, $P = 0.30$).

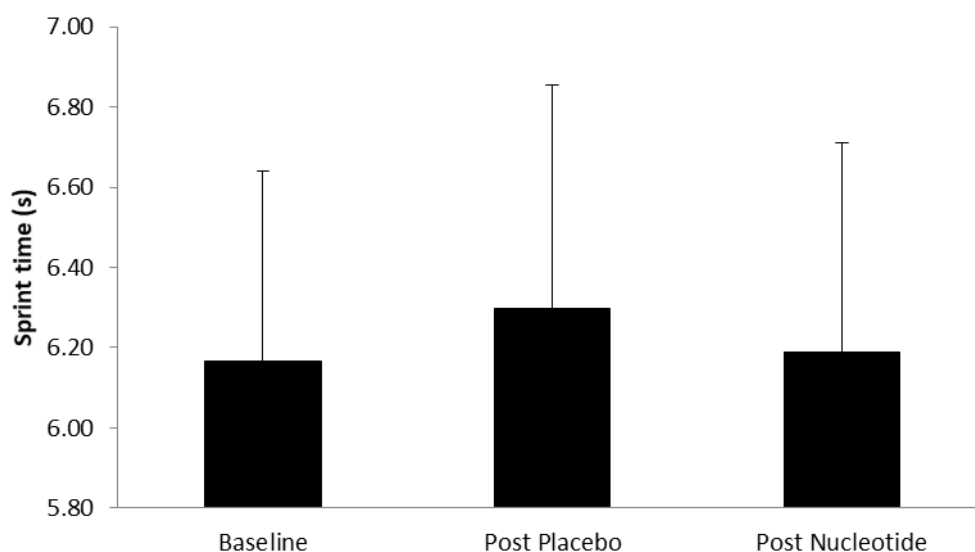


Figure 2.1.9. Average sprint time (s) for all participants over 24 sprints at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

2.4.5.3 Fatigue Index Data

During pre-supplementation phase, the mean fatigue index (%) for all 4-sets combined, was $20.9 \pm 8.9\%$ while post 30-days placebo loading were $18.0 \pm 5.9\%$ and the post 30-days nucleotide loading were $21.0 \pm 12.5\%$. There was no significant main effect or interaction effects of 30-days nucleotide supplementation upon fatigue index during pre and post supplementation ($F_{1,8, 25.1} = 1.11$, $P = 0.34$).

Table 2.2.5. Average fatigue index responses(s) for all participants across all sets at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Supplement Condition	Fatigue Index (%)				P value
	Set 1	Set 2	Set 3	Set 4	
Baseline	11.6 ± 6.3	14.5 ± 7.7	12.6 ± 5.7	10.6 ± 4.1	$p \geq 0.05$
Post Placebo	11.2 ± 4.8	12.6 ± 6.0	12.6 ± 6.8	10.3 ± 7.4	$p \geq 0.05$
Post Nucleotide	10.3 ± 6.3	14.4 ± 9.4	13.9 ± 10.9	13.4 ± 11.5	$p \geq 0.05$

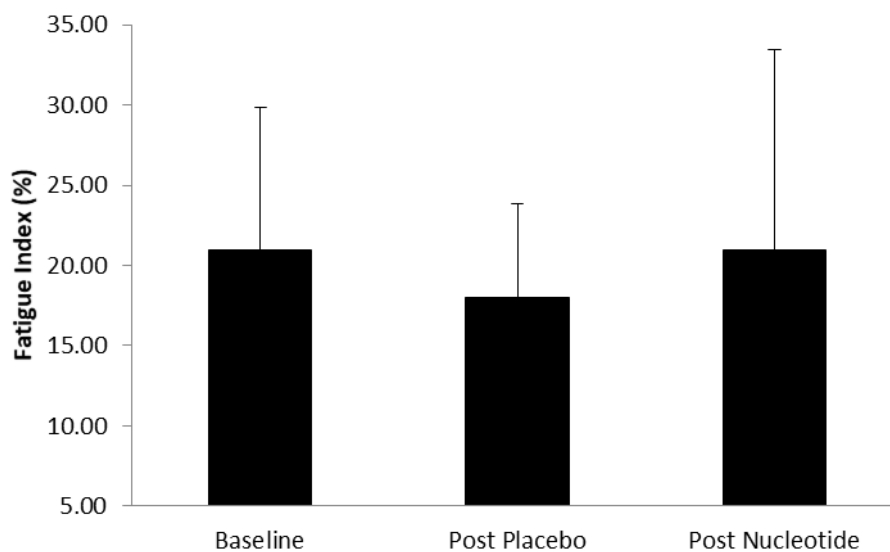


Figure 2.2.0. Average fatigue index (%) responses(s) for all participants across all 24 sprints at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

3.4.5.4 Heart Rate Data

During pre-supplementation phase, the mean heart rate for all 4-set combined, were 167 ± 12 $\text{b} \cdot \text{min}^{-1}$ while post 30-days placebo loading were 169 ± 11 $\text{b} \cdot \text{min}^{-1}$ and the post 30-days nucleotide loading were 168 ± 9 $\text{b} \cdot \text{min}^{-1}$. There was no significant main effect or interaction effect of 30-days nucleotide supplementation upon heart rate responses during sprint performance pre and post supplementation ($F_{3.0, 42.0} = 0.34$, $P = 0.79$).

Table 2.2.6. Average heart rate responses(s) ($\text{b} \cdot \text{min}^{-1}$) for all participants across all sets at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Supplement	Heart Rate ($\text{b} \cdot \text{min}^{-1}$)				
Condition	Set 1	Set 2	Set 3	Set 4	P value
Baseline	160 ± 14	167 ± 13	169 ± 12	171 ± 11	$p \geq 0.05$
Post Placebo	163 ± 11	169 ± 11	171 ± 12	171 ± 13	$p \geq 0.05$
Post Nucleotide	163 ± 11	169 ± 11	170 ± 11	172 ± 11	$p \geq 0.05$

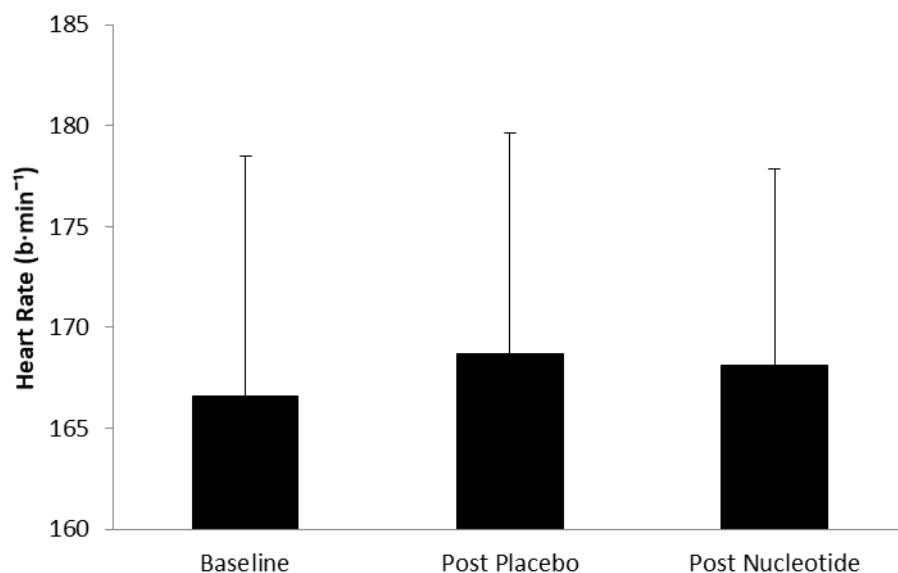


Figure 2.2.1. Mean heart rate ($\text{b} \cdot \text{min}^{-1}$) for all 24 sprints combined. Data are expressed as Mean $\pm$ SD.

2.4.5.5 Rating Perceived Exertion (RPE) Data

During pre-supplementation phase, the mean RPE for all 4-sets of repeated sprint activity combined, were 15 ± 2 while post 30-days placebo loading were 15 ± 1 and the post 30-days nucleotide loading were 15 ± 1 . There was no significant main effect or interaction effect of 30-days nucleotide supplementation upon the rating of perceived and exertion during each sprint performance pre and post placebo or nucleotide supplementation ($F_{3.7, 51.6} = 0.77$, $P = 0.54$).

Table 2.2.7. Average RPE responses for all participant's across all sets at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Supplement Condition	RPE				P value
	Set 1	Set 2	Set 3	Set 4	
Baseline	13 ± 2	15 ± 2	16 ± 2	16 ± 2	$p \geq 0.05$
Post Placebo	12 ± 2	15 ± 1	16 ± 2	16 ± 2	$p \geq 0.05$
Post Nucleotide	12 ± 2	15 ± 2	16 ± 2	16 ± 1	$p \geq 0.05$

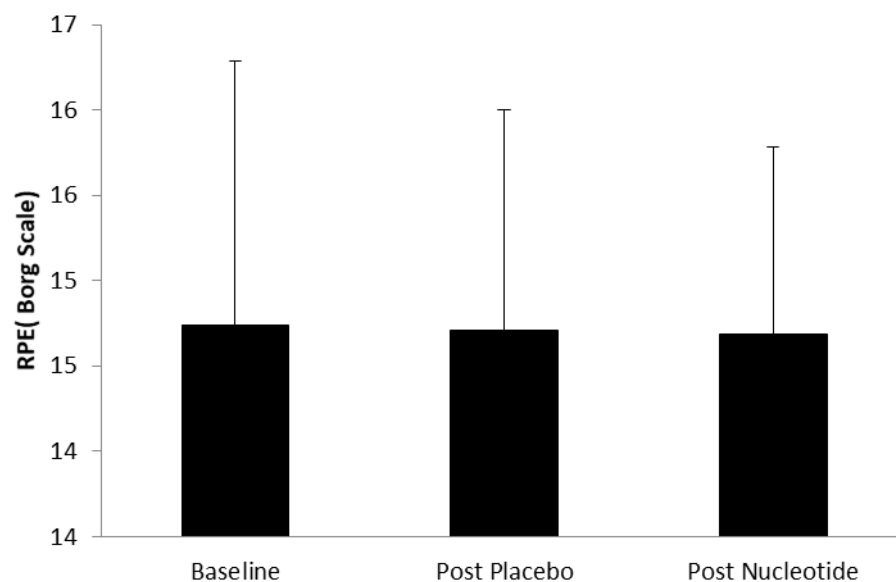


Figure 2.2.2. Average RPE (Borg, 1970) across all 24 repeated sprints combined. Data are expressed as Mean $\pm$ SD.

2.4.5.6 Blood Lactate Data

During pre-supplementation phase, the mean blood lactate for all 4-sets combined was $11.5 \pm 2.94 \text{ mmol}\cdot\text{L}^{-1}$ while post 30-days placebo loading were $10.4 \pm 1.9 \text{ mmol}\cdot\text{L}^{-1}$ and the post 30-days nucleotide loading were $10.30 \pm 1.8 \text{ mmol}\cdot\text{L}^{-1}$. There was no significant main or interaction effect of 30-days nucleotide supplementation on blood lactate responses during each set of sprint performance pre and post supplementation ($F_{2.0, 27.5} = 2.16$, $P = 0.14$).

Table 2.2.8. Average blood lactate concentration for all participants across all sets at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Supplement	Blood lactate ($\text{mmol}\cdot\text{L}^{-1}$)				
Condition	Set 1	Set 2	Set 3	Set 4	<i>P</i> value
Baseline	$9.9 \pm .4.4$	11.92 ± 3.1	11.8 ± 2.9	12.1 ± 3.2	$p \geq 0.05$
Post Placebo	10.1 ± 2.1	10.5 ± 2.5	11.6 ± 2.0	11.8 ± 2.0	$p \geq 0.05$
Post Nucleotide	9.7 ± 2.3	10.3 ± 4.9	10.6 ± 3.2	10.9 ± 2.7	$p \geq 0.05$

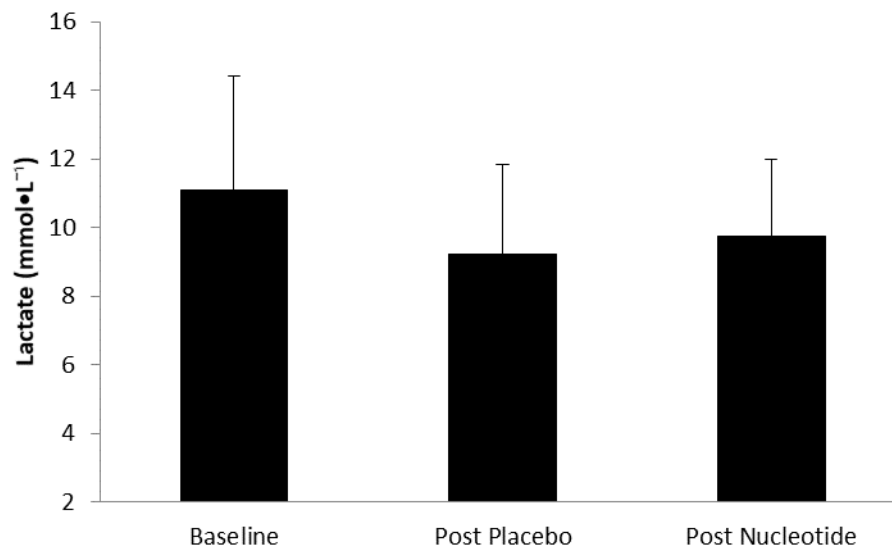


Figure 2.2.3. Blood lactate concentration ($\text{mmol}\cdot\text{L}^{-1}$) post exercise set completion averaged over all 4 sets. Data are Mean $\pm$ SD.

2.4.5.7 Uric Acid Data

The mean resting uric acid concentration for all 5 time points during pre-supplementation phase, post 30-days placebo loading, and post 30-days nucleotide loading were no significant main or interaction effects between conditions or time points for uric acid concentration ($F_{2,4, 33.8} = 0.98$, $P = 0.40$).

Table 2.2.9. Uric acid concentrations across nucleotide conditions and time following exercise. Data are expressed as Mean $\pm$ SD.

Supplement	Uric Acid ($\mu\text{mol}\cdot\text{L}^{-1}$)					
Condition	Pre	Post	Post 90 minute	Post 2 hours	Post 24 hours	P value
Baseline	230.5 $\pm$ 98.2	261.8 $\pm$ 79.0	286.8 $\pm$ 118.7	332.6 $\pm$ 136.1	235.8 $\pm$ 99.5	$p \geq 0.05$
Post Placebo	221.3 $\pm$ 81.2	255.7 $\pm$ 66.4	310.3 $\pm$ 96.5	280.5 $\pm$ 136.7	255.6 $\pm$ 104.8	$p \geq 0.05$
Post Nucleotide	220.8 $\pm$ 83.7	262.3 $\pm$ 89.9	311.6 $\pm$ 148.0	304.0 $\pm$ 143.7	209.2 $\pm$ 115.9	$p \geq 0.05$

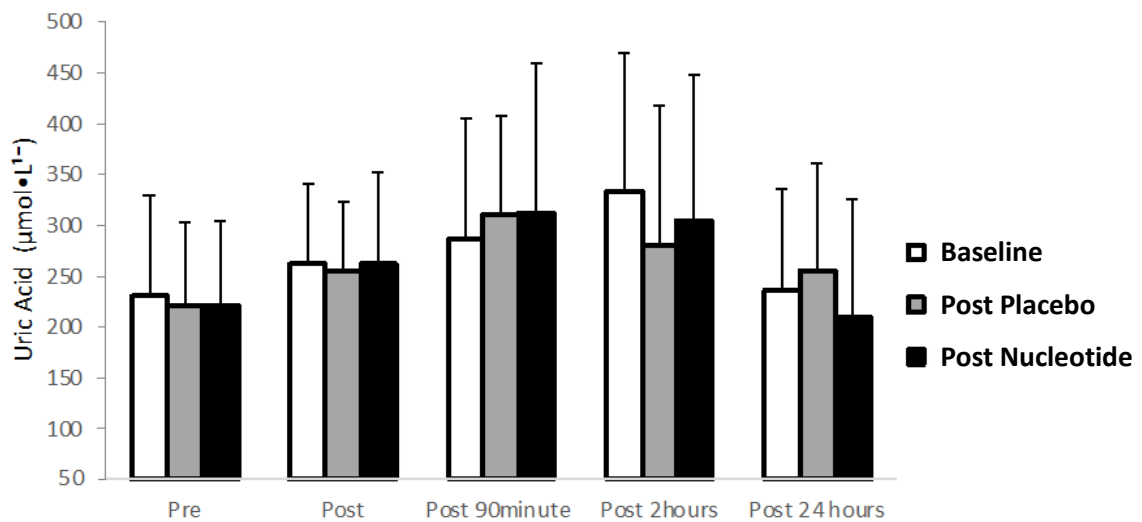


Figure 2.2.4. Mean uric acid ($\mu\text{mol}\cdot\text{L}^{-1}$) across group during different time points. Data are Mean $\pm$ SD. No Significant difference was evident ($P \geq 0.05$).

2.5 Discussion

Repeated sprint ability (RSA) requires athletes to sprint, recover, and sustain initial or near-initial performance in each subsequent sprint or series of sprints (Spencer et al., 2005; Girard et al., 2011). This chapter presents a series of performance studies evaluating the potential ergogenic effects of dietary nucleotide supplementation on RSA performance. It was hypothesised that an oral supplementation regimen of exogenous purine and pyrimidine nucleotides would enhance RSA performance, delay fatigue, and reduce physiological and perceptual strain. However, the principal findings indicate that RSA activity with exogenous nucleotide supplementation, across varying doses, supplementation durations, and experimental designs, does not show significant improvement in RSA performance, delay fatigue, or enhance physiological or perceptual responses.

The potential ergogenic effects of exogenous nucleotide supplementation are based on the hypothesis that increasing intramuscular and circulating levels of purine and pyrimidine nucleotides can expand the total adenine nucleotide (TAN = ATP, ADP, and AMP) and total pyrimidine nucleotide (TPN = cytosine, uridine, and their phosphorylated metabolites) pools. This expansion may help sustain exercise performance by enhancing the initial reservoir of salvageable nucleotides available for energy production during periods of high demand during exercise (Art et al., 1994; Yamamoto et al., 1997; Zhao et al., 2000; Gella et al., 2008; Dudzinska et al., 2010a; Alberghina et al., 2011; Xu et al., 2017; Dudzinska et al., 2018). During exercise, adenine and pyrimidine nucleotides lost or degraded in muscle cells must be restored either through the purine and pyrimidine salvage pathways [resynthesizing AMP from IMP, and UTP from UMP or uridine], or through de novo synthesis (Yamamoto et al., 1997; Dudzinska et al., 2006). Although both pathways are energetically demanding, salvage pathways are generally more efficient, requiring fewer ATP molecules for each IMP molecule resynthesised. In fact, resynthesis of IMP alone demands approximately 27 enzymatic steps and 6 ATP molecules (Dudzinska et al., 2006), while pyrimidine salvage is a shorter process of ~6 steps with lower ATP requirements (Moffatt and Ashihara, 2002). Thus, supplementation may theoretically increase nucleotide pools and reduce the energy cost of nucleotide restoration, potentially enhancing ATP availability for exercise.

Supporting this theory, previous studies have shown that inhibiting purine degradation through alterations in the expression of enzymes involved in AMP deamination (such as AMP deaminase, or AMPD) or other bioenergetic enzymes helps conserve cellular ATP (Moriwaki

et al., 1990; Gella et al., 2008; Dudzinska et al., 2010; Pospieszna et al. 2019b; Zielinski et al. 2019). Perez et al. (2004) demonstrated in a rat model that dietary purine/pyrimidine nucleotide supplementation increased hepatic nucleotide pools, altered hepatic redox state, by modulating nicotinamide adenine dinucleotide (NAD⁺) and NADH levels, and improved mitochondrial ATP turnover through enhanced TCA cycle activity. Similarly, Gella et al. (2008) observed that intramuscular pyrimidine injections promoted hepatic and muscular glucose and glycogen storage, along with increased levels of metabolic enzymes such as lactate dehydrogenase (LDH). Extending these findings, Xu et al. (2017) showed improved skeletal muscle mitochondrial function in mice through enhancement of mitochondrial enzymes activity, such as Succinic Dehydrogenase (SDH). Uberti et al. (2020) in an in vitro C₂C₁₂ myotube model found nucleotide supplementation enhanced cell signaling through PI3 kinase/ERK, improved cellular energetics [increased glucose uptake, glycogen synthesis and lactate release]. Collectively, these studies suggest potential physiological pathway(s) through which performance enhancements might occur (Ostojic, 2015).

In the present study, the focus was on the potential ergogenic effects of nucleotide supplementation as evidenced by previous performance outcomes linked to bioenergetic pathways. However, no significant improvements in repeated sprint ability (RSA) performance were observed, aligning with previous studies that found no ergogenic, physiological, or perceptual benefits following 2-minute high-intensity cycle ergometry (McNaughton et al., 2006; McNaughton et al., 2007), incremental exercise to exhaustion (Riera et al., 2013; Casajus et al., 2014), or sub-maximal endurance exercise (Coelho et al., 2021).

Contrary to these findings, some studies report ergogenic effects resulting from nucleotide supplementation. Ostojic et al. (2013) observed improved time to exhaustion during an incremental treadmill run to exhaustion after 14 days of sublingual nucleotide supplementation. Similarly, Sterczala et al. (2016) found that nucleotide supplementation attenuated the rate of isometric force loss and enhanced muscle force recovery in experienced resistance-trained athletes, although no improvements were seen in maximal isometric squat force or vertical jump performance. In the current chapter, despite varied doses, supplementation durations, and experimental designs, no improvement was observed in maximal sprint times or fatigue attenuation in RSA performance. This contrasts with Sterczala et al. (2016) and may reflect differences in exercise modality, duration, and population training status. In a rodent treadmill running model, Gella et al. (2008) found that 10 days of intramuscular pyrimidine injections

[cytosine monophosphate ($2.5 \text{ mg} \cdot \text{kg}^{-1}$; CMP) and uridine monophosphate ($3.0 \text{ mg} \cdot \text{kg}^{-1}$ UMP)] significantly extended run time to exhaustion, by over 35 minutes (~ 110 vs 75 min), while Xu et al. (2017) in a forced swimming test significantly extended swim time to exhaustion in moderate ($\sim 86\%$) and high dose ($\sim 76\%$) supplemented animals relative to low dose and placebo. Indicating a potential dose response relationship which present RSA data do not.

Variations in performance outcomes across studies may be due to several factors. Firstly, the proportion and bioavailability of purine and pyrimidine nucleotides delivered either orally or sublingually are largely unclear after each ingested dose. Dosing in the literature ranges from 50 mg per day (Ostojic et al., 2013), 480 mg (McNaughton et al., 2007), 480 mg (Riveria et al., 2013), 600 mg (Casajus et al., 2014), 278 mg (Sterczala et al., 2016) with supplementation periods varying from 14 days (Ostojic et al., 2013; Sterczala et al., 2016) to 60 days (McNaughton et al., 2007). However, no clear rationale exists for these dose choices but possibly may be determined in response to differing bioavailability estimates, depending on administration routes and subsequent biological processing (Ostojic et al., 2012; Ostojic et al., 2013). The present work proceeded with a dosing regimen predicated upon the assumption of a $\sim 2\text{-}10\%$ bioavailability profile (Coolen et al., 2011; Arts et al., 2012).

Orally delivered nucleotides face substantial degradation initially due to gastrointestinal dephosphorylation by intestinal proteases and nucleotidases, limited uptake via nucleoside transporters in the gut mucosa, gut bacteria (microbiome) uptake and high levels of first pass hepatic uptake and metabolism (Sanderson and He, 1994; Carver, 1999; Gil 2002; Baldwin et al., 2004; Hess and Greenberg, 2012; Pastor-Anglada et al., 2018; Narumi et al., 2019; Ding et al., 2021; Ding et al 2022). Although some have suggested that nucleotides could bypass degradation through passive diffusion into enterocytes (Sánchez-Pozo and Gil, 2002); however, given nucleotide size and structure, active transport via specific transporters in the enterocytes is more likely (Hess and Greenberg 2012; Bacha et al., 2013; Pastor-Anglada, and Pérez-Torras, 2018a; Pastor-Anglada and Pérez-Torras, 2018b).

Supporting this notion, Xu et al. (2017) reported an "inverted" dose-response in swim time to exhaustion, suggesting that high doses may exceed cell transporter capacity, thereby limiting cellular uptake and thus performance (Baldwin et al., 2004; Pastor-Anglada and Pérez-Torras, 2018b). As such, the estimated bioavailability of orally ingested nucleotides is likely low equating to less than 10% of initial dose ingested after 28 days (Coolen et al., 2011; Arts et al., 2012), although this bioavailability estimate is debated (Rapaport et al., 2015; Purpera et al.,

2017) and relates specifically to ATP rather than all purine and pyrimidine nucleotides, which may be preferentially transported by different classes of nucleoside transporter that may have different saturation thresholds so transport efficiency may vary (Gray et al., 2004).

If Coolen et al. (2011) and Arts et al. (2012), are correct, then at ~10% bioavailability, in best-case scenarios the oral doses used in this chapter could deliver circulating nucleotide concentration between 48 mg and 250 mg, potentially matching or exceeding the sublingual delivery levels of ~50 mg reported by Ostojic et al. (2012) and Ostojic et al. (2013). Potentially, limited nucleotide bioavailability beyond the GI tract may thus restrict any potential for an ergogenic effect on RSA performance. The impact of bioavailability on ergogenic effects is further suggested by the work of Gella et al. (2008), where intramuscularly injected pyrimidine nucleotides significantly improved treadmill endurance time in rats, and by Ostojic et al. (2012) and Ostojic et al. (2013), who demonstrated effective sublingual absorption led to improved exercise performance. Recently developed alternate modes of nucleotide delivery such as liposomal encapsulation may enhance bioavailability by allowing easier passage of nucleotide into cells, as well as protection from degradation particularly in the gut although efficacy in a human performance context remains untested. In bacteria, liposomal encapsulation has been shown to facilitate cellular ADP and ATP uptake (Narumi et al., 2019); this approach could theoretically increase cellular nucleotide bioavailability in humans through increased transport across cell membrane without specific transporters.

The variation in loading durations across studies underscores the uncertainty around the necessary duration of nucleotide supplementation for performance enhancement. The short loading periods (7 to 30 days) used in this chapter showed no performance effects with the current nucleotide formulation. The variation in loading durations presented across literature underscores the uncertainty around the necessary duration of nucleotide supplementation for performance enhancement. The short loading periods (7 to 30 days) used in this chapter showed no performance effects. Extending supplementation window, as in McNaughton et al. (2007), who used a similar nucleotide formulation but lower dose ($480 \text{ mg} \cdot \text{d}^{-1}$) for 60 days, also showed no ergogenic effects, suggesting that longer supplementation may not yield any benefits. In this chapter, nucleotide bioavailability in response to dosing levels and durations remains undetermined, leaving the critical question of whether circulating or cellular purine and pyrimidine nucleotide levels were elevated to a point that may impact performance unanswered.

Understanding the potential dose response relationships between nucleotide concentrations and loading duration is critical when assessing potential ergogenic effects. The present chapter shows that within the current RSA model across various dosages and supplementation periods; no significant ergogenic effects were observed. This lack of response suggests that the specific purine-to-pyrimidine nucleotide concentrations and ratios tested do not influence RSA performance or related physiological and perceptual responses. In contrast, animal models have demonstrated a range of beneficial biological effects, including enhanced exercise performance and metabolism, at nucleotide doses in the hundreds to thousands of $\text{mg}\cdot\text{kg}^{-1}$ of body weight (Xu et al., 2017; Li et al., 2019; Ridwanudin, et al., 2019; Ding et al., 2022). For instance, the doses used by Xu et al. (2017) in rats would translate to approximately 11,200 mg and 44,800 $\text{mg}\cdot\text{kg}^{-1}$ day for a 70 kg human, depending on the intervention level. This exceeds the $\text{mg}\cdot\text{kg}^{-1}$ body weight used in the high-dose protocol of this chapter and the suggested safe upper absolute exposure limit of 4,620 mg per day for humans, equivalent to $\sim 66 \text{ mg}\cdot\text{kg}^{-1}$ body weight (Verkerk and Gil, 2009). To explore potential ergogenic effects, it may be necessary to substantially increase nucleotide doses toward the thresholds for safe humans' exposure $\sim 66 \text{ mg}\cdot\text{kg}^{-1}$ (Verkerk and Gil, 2009). However, this approach requires caution. Recent studies have linked high dietary nucleotide intake (e.g., $\sim 2,000$ mg per day from mycoprotein supplementation) with hyperuricemia after only seven days of supplementation, leading to clinical recommendations to limit daily nucleotide intake to below $\sim 2,000$ mg to mitigate this risk (Coelho et al., 2020; Coelho et al., 2022).

Increasing the nucleotide dose and duration may impact ergogenic responses, but the purine-to-pyrimidine nucleotide ratio in supplementation formulations could also be a critical factor for ergogenic effects. In both in vivo and in vitro studies, Xu et al. (2017) and Uberti et al. (2020) observed distinct adaptations in vivo and in vitro in mitochondrial enzyme concentrations, energy metabolism, and antioxidant responses in rodent exercise models and C₂C₁₂ myotube models, respectively, depending on the nucleotide formulation used. Uberti et al. (2020) found that C₂C₁₂ myotube responses varied according to the specific nucleotide composition, a trend also noted in animal studies on disease resistance (Hess and Greenberg, 2012; Ding et al., 2021). These findings suggest that different purine and pyrimidine formulations may influence distinct physiological pathways based on their relative concentrations. Thus, various nucleotide formulations may elicit diverse effects in vivo and in vitro exercise models, depending on concentration and nucleotide composition (Xu et al., 2017; Uberti et al., 2020). The supplementation of single purine nucleotides such as ATP can produce

ergogenic effects across multiple activities supports this hypothesis; these effects typically occur at doses higher than those used in the present studies (>400 mg) (Jordan et al., 2004; Jager et al., 2021). The intramuscular delivery of CMP alone also delivers ergogenic effects in run time to exhaustion (Gella et al., 2008). More, recently Nakagawara et al. (2022) has observed both 5'-CMP and 5'-UMP enhance muscle cell growth and mitochondrial biogenesis. In contrast, supplementation with other nucleobases (e.g., adenine, guanine, inosine) and ribose [a key component in nucleotide de novo synthesis and salvage pathways] has shown limited or no efficacy in enhancing performance (Op't Eijnde et al., 2001; Kavazis et al., 2004; Kersick et al., 2005; Peveler et al., 2006; Coelho et al., 2021).

Direct comparisons between the current performance data and findings from animal, human, and in vitro studies are challenging due to differences in dosing regimens, purine-to-pyrimidine ratios, and the exceeding of safe dosage thresholds for human administration needed to replicate the animal work. Additionally, the studies presented in this chapter utilised a fixed dose across participants with varying body masses, leading to differences in ingested amounts per kg of body weight. This variability must be acknowledged as a limitation of the supplementation approach, particularly in studies employing cross-sectional designs.

Moreover, concerns arise regarding the reliability of the performance tests used in studies reporting ergogenic effects. Ostojic et al. (2013) evaluated performance using a stepwise, incremental treadmill time-to-exhaustion (TTE) test, which has been criticised for poor reliability (Laursen et al., 2007; Currell and Jeukendrup, 2008). Although Ostojic et al. (2013) reported high correlations following familiarisation, correlation does not equate to reliability, and the observed ~15-second (~5%) improvement could reflect motivational factors, measurement error, or biological variation (Katch et al., 1982). Similarly, Sterczala et al. (2016) did not report reliability data for any strength or power performance measures and similar influence on performance as noted above are possible. Interpretation of this study is further complicated by the inclusion of additional compounds (L-glutamine, L-methionine, L-lysine, riboflavin, folate, biotin, inositol, sodium citrate) in the nucleotide supplement (Nu-Bound), confounding the attribution of observed effects solely to nucleotides.

In the rodent TTE forced swimming model used by Xu et al. (2017), a significant extension in swim performance was noted, but reliability data for the TTE test was not provided. This lack of reported reliability data, combined with behavioural factors that impact TTE and exercise intensity in the tail-weighted swimming model, complicates the interpretation of these findings

(Bogdanova et al., 2013; Dos Reis et al., 2018). While similar methodological concerns may apply to the rodent TTE model of Gella et al. (2008), the substantial ~35-minute improvement in TTE suggests a strong biological effect from intramuscular pyrimidine supplementation, warranting further exploration particularly through in vitro ‘twitch induction’ skeletal muscle models.

To investigate the ergogenic effects of chronic oral nucleotide ingestion, we employed a Repeated Sprint Ability (RSA) performance model, which contrasts previously used single high-intensity, endurance, and strength exercise protocols tested in both animal and human models (McNaughton et al., 2006; McNaughton et al., 2007; Gella et al., 2008; Ostojic et al., 2013; Casajus et al., 2014; Sterczala et al., 2016; Xu et al., 2017). In this RSA model high-repeated intensity exercise, deplete purine nucleotide pools in skeletal muscle by ~ 21-45%, primarily via the pathway ($\text{ATP} \rightarrow \text{ADP} \rightarrow \text{AMP} \rightarrow \text{IMP}$). Typically, in skeletal muscle, purine degradation typically stops at inosine 5'-monophosphate (IMP), as it is the final intracellular step for energy metabolism; being non-membrane permeable, it is retained in the muscle. After exercise, when energetic demand is reduced, IMP is re-aminated and re-phosphorylated to AMP/ADP/ATP during recovery, a process that completes within approximately 10 minutes following a single 30-second Wingate sprint (Zhao et al., 2000). However, during repeated high-intensity exercise with increased metabolic requirements, the AMP/ATP ratio rises, further converting AMP to IMP. A portion of intracellular IMP is further dephosphorylated by 5'-nucleotidase, to maintain ATP turnover, to the degradation products inosine, hypoxanthine, which then diffuses out of the muscle into circulation, leading to a net loss of purine nucleotides, as these products cannot re-enter the muscle (Tullson et al., 1995; Dudzinska et al., 2010b; Ipata et al., 2011). Circulating hypoxanthine is then converted to uric acid in the liver, with most of it subsequently excreted (Hellersten et al., 1998; Hellersten-Westling et al., 1994; Johnson et al., 2021). Although we did not assay direct markers of purine metabolism (ATP, ADP, AMP, inosine, hypoxanthine) or pyrimidine degradation (uridine), elevated uric acid indirectly indicates purine metabolism during intense exercise (Jamurtas et al., 2018; Johnson et al., 2021). In the present studies (studies 1 and 2), uric acid levels increased, but did not significantly differ post-RSA protocol in either supplemented, or placebo conditions, suggesting ATP demand was met without extensive purine degradation leading to purine loss from the muscle, pointing to the potential activation and contribution of other metabolic pathways. Similarly, Dudzinska et al. (2010a) noted that continuous high-intensity exercise results in the simultaneous degradation of pyrimidine nucleotides, due to the reduced

rate of UDP phosphorylation to UTP, as UDP is used as a phosphate donor to facilitate the re-phosphorylation of AMP/ADP/ATP when cell energy status may be compromised (Connolly and Duley, 1999; Yamamoto et al., 2011). This pathway accelerates pyrimidine degradation (UTP $\rightarrow$ UDP $\rightarrow$ UMP $\rightarrow$ Uridine $\rightarrow$ Uracil), resulting in increased UMP and uridine concentrations immediately and 30 mins post intense exercise (Yamamoto et al., 1994; Yamamoto et al., 1997; Ohno et al., 2008; Dudzinska et al., 2010b). The provision of a pyrimidine formulation in this work did not improve any performance metric and the lack of degradation product assay to indicate the activation and importance of this pathway in enhancing or maintaining RSA exercise activity remains unresolved.

A clear rationale for the lack of nucleotide efficacy with RSA exercise remains undetermined, however the nature of the RSA protocol and the individual responses to that undertaken would seem central to the responses noted. RSA exercise duration, intensity, repetition count, recovery time between repetitions and sets, and the nature (active vs passive) of that recovery all may impact nucleotide metabolism and efficacy through manipulation of the different energy system contributions to the maintenance of ATP turnover (Boobis et al., 1982; Gaitanos et al., 1993; Dawson et al., 1997; Hellsten et al., 1998; Connolly et al., 2003; Skotnicka et al., 2010; Mendez-Villanueva et al., 2012; Gharbi et al., 2014; Hargraves and Spriet, 2020; Ulupinar et al., 2021). Firstly, these protocol factors likely influence the relative balance of aerobic and anaerobic energy systems contribution during each repetition and between set(s) to support ADP/ATP turnover (Spencer et al., 2005; Glaister et al., 2006; Gharbi et al., 2014; Ulupinar et al., 2021). The nature of the current RSA protocol with shorter duration (~6 s) repeated high intensity bouts (6 x 35 m sprints, 15-s active recovery [$\sim 2.3\text{m}\cdot\text{s}^{-1}$], 5-min passive rest between set recovery) preferentially deplete phosphocreatine (PCr) stores, initially to support ADP/ATP turnover followed by glycolysis and oxidative phosphorylation contributions. This protocol structure may attenuate the rate and/or the amount of purine degradation to hypoxanthine that occurs due to a reliance on PCr and glycolytic energy supply rather than bringing about more complete purine degradation that would be evidenced in elevated uric acid levels; an outcome that was not observed. These observations are in line with those of Bogdanis et al. (2022) who noted that longer durations of high intensity exercise (60s) rather than shorter (10s) are required to impose a sustained demand on ATP resynthesis, which would lead to a more complete degradation and loss of muscle purine levels. In a similar RSA protocol (6 x 35 m sprints), Milioni et al. (2017) reported energy contributions of approximately 28% from phosphagen, 34% from glycolytic, and 38% from aerobic pathways.

Likewise, Ulupinar et al. (2021) found that during a 30-m sprint RSA protocol (x10 repetitions), about 60% of energy was derived from non-oxidative sources, of that 47% was estimated from phosphagen and 13% from glycolytic pathways. Together, these findings collectively suggest a substantial contribution from both non-oxidative (~60%) and oxidative (~40%) sources in sustaining RSA performance. Elevations in current post exercise blood lactate concentration ($\sim 9\text{--}13\text{ mmol}\cdot\text{L}^{-1}$) would serve as a broad indicator of a substantial non-oxidative contribution to RSA metabolic activity, along the lines of those noted above (Baker et al., 2010; Dardouri et al., 2014; Gharbi et al., 2014).

The timing and nature of the work-to-rest ratios executed in the RSA protocol provides an important function in dictating metabolic responses (Bogdanis et al., 2021; Ulupinar et al., 2021). Short rest intervals ($<10\text{ s}$) during repeated sprints allow the partial replenishment of PCr stores, removal of accumulated inorganic phosphate, ammonia, and restoration of muscle pH towards resting values (Bogdanis, et al., 1996; Bishop et al., 2004; Bogdanis et al., 2021). Such intervals may be either active or passive or a combination of both as in the current studies however, whether they are passive or active impacts these RSA exercise restoration processes (Spencer et al., 2005). Gaitanos et al. (1993) noted that 30-s passive recovery intervals between consecutive sprints were sufficient to allow significant PCr resynthesis and, as a result, contribute to the maintenance of ADP/ATP turnover and exercise performance. Further, Dawson et al. (1997) reported after 5 x 6 s sprints with 30-s passive recovery PCr depletion of muscle creatine to 27% of resting 10 seconds after completion and returned to 87% of pre-exercise values at 3 minutes post completion. Mendez-Villanueva et al. (2012) report that muscle PCr concentrations are depleted to $\sim 47\%$ of the resting value after 10 x 6-s cycle ergometer sprints with 30-s passive rest intervals and resynthesised to $\sim 85\%$ of the pre-exercise concentrations after 6 min of passive recovery. Further, Ulupinar et al. (2021) reported that ATP-PCr concentrations were replenished at the highest rate in the initial period of passive recovery ($\sim 15\text{--}20\text{ s}$) following RSA activity. It must also be acknowledged that the extent of these metabolic responses/changes will respond as a function of their background training history and sport specific energy system adaptations.

Conversely, in the current protocol an active recovery was implemented between sprint repetitions. Spencer et al. (2006) observed impaired PCr restoration $\sim 21\text{-s}$ after active (55% restored) relative to passive recovery intervals (72% restored). Spencer et al. (2008) further indicated that performance [peak power] during repeated-sprint tests was impaired after

moderate and low intensity active recovery trials, relative to passive. Buchheit et al. (2009) and Billaut and Buchheit, (2013) have shown that within set active recovery relative to passive recovery decreases muscle tissue oxygenation during shuttle running activity. Thus, the short active recovery intervals (~15-s) as implemented between the sprints in this chapter could act to attenuate the resynthesis of PCr, glycolytic pathway activation and the recovery of performance during each subsequent sprint as a function of reduced muscle tissue oxygenation/availability (Dupont et al., 2004; Ohya et al., 2013; Brown and Glaister, 2014; Kriel et al., 2016; Madueno et al., 2019; Fennell et al., 2021; Turner et al., 2021). This may explain the increase in sprint times during each set from sprint 1-6. Whilst within set decreases in sprint performance are apparent, the lack of a significant decline in average performance and associated outcome variable over the course of the whole protocol performance from sets 1-4 could be explained by the 5 min passive recovery phase implemented between RSA sets. Several studies have noted that this timeframe is sufficient to allow a comprehensive restoration of PCr and available purine metabolites for use during subsequent exercise sets and restore muscle pH towards resting levels (Harris et al., 1976; Balsom et al., 1992; Bogdanis et al., 1996; Dawson et al., 1997; Zoladz et al., 2010). Barbosa et al. (2021) have similarly reported that an even shorter period of ~4 minutes of passive recovery is sufficient to restore repeated sprint exercise performance. This may confound the expression of any ergogenic nucleotide effect.

Restoration of RSA performance is correlated highly with individual $\dot{V}O_2$ (Rampinini et al., 2009; Buchhiet et al., 2010; Billaut and Buchheit, 2013). A positive relationship between aerobic fitness and the fatigue profiles exhibited during RSA activity is indicated (Bishop et al., 2003; Bishop and Spencer, 2004; Glaister et al., 2005; Buchhiet et al., 2010). This agrees with the strong correlation observed ($\sim r$ 0.86; $P < 0.001$) between $\dot{V}O_2$ max and RSA performance [not shown] within the participants presented in the current chapter. Higher individual aerobic fitness levels may contribute to a reduction in the need for glycolytic pathway activation and act to ensure optimal PCr resynthesis during the recovery periods between each sprint and by allowing increased oxidative contribution to complete restoration of PCr and performance recovery during the 5 minute passive recovery phases between set (Mc Mahon and Jenkins 2002; Mendez-Villanueva et al., 2012; Kalva et al; 2017; Milinoi et al., 2017; Ulupinar et al., 2021). Given the heterogeneity in aerobic fitness of the individuals within each population in this chapter, this may be an important factor contributing to the lack of nucleotide ergogenic effects by allowing individual endogenous nucleotide pathways to meet their individual nucleotide demand without recourse to exogenous nucleotides as a nucleotide

‘top up.’ Thus, a potential reason as to why there were no observed differences in outcome variables between supplemented and un-supplemented conditions. These effects were, however, mitigated because equally across all the conditions presented in this chapter as participants were allocated to each conditions using a minimisation of difference protocol based upon $\dot{V}O_2$ max.

It has been argued that nucleotides may also act to lower lactate concentrations (Koeppel, 2011). Nucleotides could regulate glycolytic rate by allosterically modulating phosphofructokinase (PFK) activity, a rate-limiting enzyme in glycolysis when the ATP: AMP ratio is too high in favour of ATP (Larsson et al., 1997). Elevation of adenine nucleotide levels through via supplementation may potentially inhibit PFK, down-regulating glycolysis and slowing the conversion of fructose -6- phosphate to fructose-1-6-biphosphate thus slowing glycolytic flux; potentially reducing lactate appearance and changes in muscle pH, particularly during RSA tasks (Berg et al., 2002; Koeppel, 2011; Brüser, 2012). Likewise, pyrimidine nucleotides might also influence glycolysis rate, providing intermediates for the TCA cycle and increasing cellular glucose and glycogen stores (McNaughton et al., 2007; Gella et al., 2008; Alberghina et al., 2011). Gella et al. (2008) demonstrated that intramuscular pyrimidine supplementation down regulated liver LDH catalytic activity, and significantly attenuated exercise induced elevation in liver but not muscle lactate. However, they noted significantly lower muscle lactate after a single bout of 5-minute treadmill exercise relative to un-supplemented animals. It may be that expansion of the TPN pool was sufficient to alter glycolytic flux and or drive cellular glucose higher altering metabolic responses (Gella et al., 2008). Similarly, Xu et al. (2017) reported a dose-dependent reduction in blood lactate attributed to an increase in mitochondrial enzymes and function after a forced swim test in animals following nucleotide supplementation relative to placebo control. However, contrary to these previous animal studies and mechanisms outlined above, nucleotide supplementation did not significantly affect lactate levels post-RSA, supporting previous exercise study findings in humans by McNaughton et al. (2007) and Riera et al. (2013), who observed no change in post-exercise lactate relative to controls after prolonged (30-60 days) nucleotide supplementation in humans.

Nucleotides may exert ergogenic effects upon exercise performance through other mechanisms, such as acting on purinergic receptors to regulate vascular tone, blood flow and enhance oxygen transport in muscle tissue capillary beds (Hellsten et al., 1998; Rosenmeier et al., 2008; Ellsworth, 2016; Yegutkin, 2021). More specifically, improved peripheral (muscle) oxygen

transport may occur, possibly via improved erythrocyte count (Hb concentration) and/or alterations in the oxy-haemoglobin disassociation curve from increased erythrocyte concentrations of 2,3 diphosphoglycerate, improving the availability of oxygen to peripheral tissues (Art et al., 1994; Scopesi et al., 1999; Carver et al., 2002; Rosenmeier et al., 2008; Yousefi et al., 2011). However, these observations were not replicated in a follow up human infant study, perhaps reflecting the 30-fold higher concentration of nucleotide administered in the rodent model relative to human work (Scopesi et al., 2006). As such efficacy seems to be expressed where levels of exogenous supplementation are delivered at a higher level than that permitted in humans. Further verification of these pathways potentially by NIRS muscle tissue oxygenation assessment during RSA exercise after nucleotide supplementation is required.

Several limitations may have impacted the studies outcomes. Although the combined sample size in the dose response study (study 1 was large (n=43), a G power analysis indicated that detecting statistically significant differences with the observed effect size of (0.03) and an alpha of 0.05 and power of 80% would require impractically large sample sizes. Given such a small effect size on RSA activity in this design and population, such an experiment is not feasible. Taking a within subject repeated measures design the sample estimates decline but represent an unfeasible sample size. Additionally, participants were required to self-verify nucleotide ingestion and adherence to the dosing ingestion protocol and schedule at specified times throughout the day. Although nucleotide supplement return and pill count were requested for unused product, not all participants complied fully, which may have led to potential inconsistencies in verification of compliance with nucleotide consumption. Additionally, adherence to pre-testing exercise abstinence was confirmed through verbal verification only, limiting the ability to accurately assess compliance rates.

The participants recruited into each study, presented with varied training backgrounds, including recreational and trained individuals across strength, endurance, and intermittent exercise disciplines. This individual heterogeneity, especially in the cross-sectional studies, may have influenced the specificity of performance and nucleotide responses on the RSA task, which was familiar to some participants but novel to others. Prior studies have shown that trained individuals, particularly those in sprint disciplines, exhibit reduced purine nucleotide loss and enhanced purine salvage compared to endurance-trained or untrained individuals (Skotnicka et al., 2010; Zielinski et al., 2013). Additionally, endurance-trained individuals across a broad age spectrum (20-90 years) demonstrate improved purine/pyrimidine

metabolism, reduced nucleotide loss and better recovery of nucleotide pools compared to untrained individuals (Zielinski et al., 2019; Pospieszna et al., 2019; Kistner et al., 2021; Pospieszna et al., 2021). This hierarchy [sprint-trained > endurance-trained > untrained] suggests that individual training status (training adaptation) influences nucleotide loss and purine recovery efficiency and may contribute to the lack of effect observed in the groups across the current chapter. The observed performance outcomes may thus be partly attributed to the individual participants' diverse training backgrounds, which affected their nucleotide conservation and recovery abilities. Future research should prioritise a more homogeneous sample population in terms of athletic background and fitness status, potentially matched on hypoxanthine (Hx) and Hx-guanine phosphoribosyltransferase (HGPRT) levels as these variables provide a superior discriminatory function of muscle nucleotide metabolism than $\dot{V}O_2$ max (aerobic capacity) which was used in the present work to allocate participants to group (main study 1). Finally, the RSA protocol involved 24 sprints, organised into 4 sets of 6 sprints each. A potential limitation of this protocol setup is that it may have led some participants to adopt a conscious or unconscious pacing strategy, as observed in other studies of repeated maximal sprint protocols (Fitzsimmons et al., 1995; Spencer et al., 2005). To minimize this effect, each set was limited to 6 sprints, followed by a 5-minute passive recovery (Chaouachi et al., 2010). However, individual data points suggest that pacing may still have occurred, as some participants' final sprint repetitions were as fast as their initial ones. While the recovery period might explain some of this variation, pacing is likely to have influenced certain performances although attribution and identification is problematic.

2.6 Conclusions

The execution of repeated sprint ability is a complex fitness component that depends on the interaction of metabolic (e.g., ATP (re) synthesis, phosphocreatine recovery, oxidative capacity, and H⁺ buffering) and neural factors (e.g., muscle activation and recruitment strategies) (Buchheit et al., 2010; Girard et al., 2011). The studies presented in this chapter sought to expand total adenine and pyrimidine nucleotide cellular concentration via the ingestion of oral purine and pyrimidine nucleotide supplement. Despite several potential mechanistic pathways of action, no significant effect on RSA performance and associated psycho-physiological markers was determined. The case for oral nucleotide supplementation as an RSA ergogenic aid remains unproven in humans despite some interesting *in vivo* animal data. This observation

must be considered in light of oral nucleotide composition, dosing requirement, nucleotide bioavailability and critically appropriately powered studies based upon these considerations. It further, remains to be determined whether the efficacy of nucleotides as an ergogenic aid in humans may reside outside performance improvement but may act as an adaptogenic agent mediating cell protection recovery and regulation of immunity and cellular stress as postulated by others in animal and human stress models. The future studies within this thesis will examine these avenues.

Chapter 3

Effect of 30-Days Nucleotide Supplementation upon Gastrointestinal Permeability, Damage, and Symptomology in Response to Acute Repeated Sprint Exercise. (Study 2)

Despite the extensive evaluation of the various nucleotide dose and duration supplementation models upon RSA (Repeated Sprint Ability) performance, no ergogenic effects were observed across the interventions studied. This consistent lack of performance enhancement suggests nucleotide dose and formulation may have limited applicability in this specific high-intensity RSA context subject to the caveats considered in the previous chapter. However, the potential benefits of nucleotide supplementation may not be entirely performance-based. Rather than acting directly as an ergogenic aid, nucleotides may exert adaptogenic effects, supporting systemic recovery and resilience under and following exercise mediated physiological stress. These properties open the door to a broader interpretation of exercise performance support; one that includes optimising physiological system function. In particular, the gastrointestinal tract plays a critical role in nutrient absorption, immune modulation, and facilitating exercise performance. High-intensity exercise often compromises GI integrity, leading to gastrointestinal dysfunction, adverse symptomology, and compromised performance. Animal and human model research suggests that nucleotides may enhance gastrointestinal mucosal repair thus maintaining gut barrier structural integrity and function. Therefore, the following chapter will explore the role of chronic nucleotide supplementation upon GI function following high-intensity RSA activity in a recreationally active human model. This shift in focus may reveal an indirect pathway to enhancing performance through nucleotide effects on gastrointestinal permeability, damage, and symptomology.

3.1 Abstract

This chapter examined the effects of the oral purine and pyrimidine nucleotides administration upon GI permeability and symptomology after high intensity repeated sprint exercise. Fifteen male recreationally trained individuals [age, 21.7 ± 4.2 years; height, 177.7 ± 5.0 cm; weight, 72.4 ± 6.7 kg; $49.0 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$] undertook in a randomised double blind counterbalanced format three trials of high intensity intermittent repeated sprint exercise (RSA) [4 set x [6x35 m @ 15 sec active recovery] with a 5 min passive recovery between sets. Each participant undertook 1. Exercise as baseline assessment (BExe) 2. Exercise following 30 days nucleotide ingestion (2520mg) (NExe) and 3. Exercise with 30 days placebo ingestion (2520mg) (PExe). Ingestion of nucleotide supplement was stopped on the night prior to RSA protocol commencement. The ratio of Lactulose/L-rhamnose (L/R) appearance in serum was used to reflect changes in GI permeability, Intestinal fatty acid binding protein (IFABP) was used to reflect epithelial GI damage, symptomology score was determined with the GI discomfort scale. Heart rate, lactate, RPE, thermal comfort and tympanic temperature and sprint performance times were collected. Serum biomarkers of gut permeability were determined via HPLC and gut damage (IFABP) via ELISA. Serum cortisol was utilised as a marker of stress responses via ELISA. Data indicate despite exercise performance equivalence across all conditions (NExe 6.19 ± 0.52 s vs PExe 6.30 ± 0.56 s vs BExe 6.17 ± 0.47 s; $P \geq 0.05$) that GI permeability (L:R ratio) was not significantly affected (NExe 0.029 ± 0.02 vs PExe 0.018 ± 0.01 vs BExe 0.012 ± 0.01 ; $P \geq 0.05$). Similarly, no significant differences in IFABP (NExe 440.6 ± 104 pg/mL vs PExe 376 ± 70 pg/mL vs BExe 468 ± 151 pg $\cdot$ mL $^{-1}$; $P \geq 0.05$) or subjective GI symptomology which was either absent or very mild was apparent between conditions. These data suggest that nucleotide supplementation for a period of 30 days following the 2520mg dosing regimen in recreationally active males had no significant effects on GI permeability, damage and symptoms following the performance of a high intensity repeated sprint protocol. Further, no attenuation in stress response (cortisol) across exercise conditions was evidenced.

3.2 Introduction

The gastrointestinal tract has a central role in nutrient absorption (Pfeiffer et al., 2009; van Wijck et al., 2013), regulation of immunity (Valdés-Ramos et al., 2010; Mu et al., 2017; Burgueño, and Abreu, 2020; Ding et al., 2021), inflammation (Somsouk et al., 2015; Bander et al., 2020) and hosting a diverse microbiota population (Fung, 2020) all of which play a central role in human health and wellbeing. The Gastrointestinal barrier collectively describes the physical, cellular, and chemical properties of the intestinal mucosa; a multi-layered barrier that maintains the integrity of gastrointestinal tract by allowing the establishment of a selectively permeable barrier that supports nutrient absorption whilst preventing the adherence and movement of pathogenic/antigenic agents from the gastrointestinal lumen into the systemic circulation (Lambert, 2008; Turner, 2009; Bischoff, 2011; Fasano, 2011; Camilleri et al., 2012; Grootjans et al., 2016; Camilleri, 2019; Camilleri and Veld, 2021). Nomenclature to describe the loss of gastrointestinal barrier integrity in the literature is varied, the terms gastrointestinal permeability (Lambert, 2008; Marchbank et al., Pugh et al., 2017); leaky gut (Fasano, 2011; Vojdani, 2013; Camilleri, 2019; Paray et al., 2020; Camilleri and Vella, 2021), gut damage (Playford et al., 1999; Costa et al., 2017; March et al., 2017) have been reported and used interchangeably to describe adverse changes in gut function. However, each describes a distinct process and is likely to have different mechanistic pathways, time sequences of activation, progression, and physiological outcomes (Costa et al., 2017). Gastrointestinal permeability the primary clinically verified approach refers to the translocation of low molecular weight particles through the mucosal and endothelial membranes of the gastrointestinal barrier via paracellular and/or transcellular pathways (Lambert, et al., 2002; Fasano and Shea-Donohue, 2005; Fehrenbach and Schneider, 2006; Lambert et al., 2008; Selkirk, et al., 2008; Tripathi et al., 2009; Vojdani, 2013; Paray et al., 2020). Determination of gastrointestinal permeability is assessed by the urinary and/or serum concentrations of orally administered ‘sugar probes’ (Fleming et al., 1996; Camilleri et al., 2012; Vojdani, 2013; van Wijck et al., 2012; van Wijck et al., 2013). Using a combination of dual ‘sugar’ probes that vary in molecular weight [size] the technique provides an indication of intestinal integrity by determining permeability changes in the small and large intestines (Lambert, 2008; Vojdani, 2013; van Wijck et al., 2013). A disaccharide (usually lactulose) and a monosaccharide (L-rhamnose, mannitol or D-xylose) sugar probe are typically combined (Lambert et. al., 2008; van Wijck et al., 2013; Pugh et al. 2017; Costa et al., 2017). Lactulose (molecular weight=342 Da) is assumed to only be absorbed paracellularly when intestinal barrier function is

compromised whereas L-rhamnose, (molecular weight= 164 Da), is considered to cross the intestinal barrier via a transcellular route (Lambert et al., 2008; Vojdani, 2013; Bischoff et al., 2014). Permeability is expressed as the ratio of the large molecule (s) to the small molecule (s) appearance over a defined collection period (Fleming et al., 1996; van Wijck et al., 2011; van Wijck et al., 2012). Urinary recovery of these ‘sugar probes’ has been the gold standard however others have sought to modify the assay process (Fleming et al., 1996; van Wijck et al., 2011). Blood derived permeability analysis has more recently been implemented to assess intestinal permeability over a shorter time period usually 2 h to improve experimental data collections procedures and compliance (Fleming et al., 1996; van Wijck et al., 2012; van Wijck et al., 2013; Pugh et al., 2017).

Other biomarkers have been reported to represent ‘damage’ to gastrointestinal barrier integrity; the most prominent reported in literature is Intestinal Fatty Acid Binding Protein [I-FABP] (van Wijck et al; 2012; Davidson et al., 2016; Marchbank, et al., 2017; Snipe, 2017; Pugh et al., 2017). I-FABP is a small cytosolic protein located in the small intestinal apical from the villus tips, which is released into the circulation upon cellular damage. This has been the most widely applied biomarker in the exercise setting typically as a key biomarker of enterocyte tissue damage (van Wijck et al., 2012; Costa, et al; 2017; Pugh et al., 2017; Chantler et al., 2021). This differentiates gastrointestinal villus damage from gut permeability where the tight junction proteins are disrupted (Pugh et al., 2017; Costa et al., 2017; Chantler et al., 2021). The mechanism of increased gastrointestinal permeability has been associated with disruption to enterocyte tight junction protein expression and the subsequent impairment of basement membrane barrier function (Fasano and Shea-Donohue, 2005; Fasano, 2012; Dokladny et al., 2013; Zuhl et al., 2014). Typically, tight junction proteins maintain enterocyte structural integrity both within and between adjoined cells by anchoring each cell together (Turner, 2009; Grootjans et al., 2013; Zuhl et al., 2014; Grootjans et al., 2016). Mucosal injury because of these factors can initiate an inflammatory state and further alter tight junction connectivity. Consequently the magnitude of the transcellular and paracellular translocation of gastrointestinal luminal antigens into systemic circulation may increase leading to further immune activation and gastrointestinal mucosal inflammation (Heumann and Roger 2002; Dokladny, et al., 2008; Tripathi et al., 2009; Fasano, 2012; Dokladny et al., 2013; Zuhl et al., 2014; Colgan et al., 2016; March et al., 2017; Snipe et al., 2018; Camilleri and Velda, 2021; Ding et al., 2021). The ability of the gastrointestinal barrier to fulfil its role(s) can be compromised by a range of psycho-physiological stressors i.e., disease, physical exercise,

thermal stress, pathogens, hormonal disruption and pharmacological agents that may breach this integrity and cause a range of both objective and subjective symptoms (Ashton et al., 2003; Costa et al., 2017; Grootjans et al., 2013a; Grootjans et al., 2016; Shulman et al., 2014; Marlicz et al., 2014; Vieth and Montgomery, 2017).

Epidemiological and experimental data report increased subjective symptomology of gastrointestinal dysfunction (e.g., nausea, bloating wind, diarrhoea, abdominal pain) in both male and female athletes following exercise (Riddoch and Trinick, 1988; Moses, 1990; Lambert et al., 1999; Peters et al., 1999; ter Steege et al., 2008; ter Steege, et al., 2012; Haaf et al., 2014; de Olivera et al., 2014; Wilson, 2017). Endurance athletes seem particularly susceptible, expressing subjective gastrointestinal symptoms occurrence frequencies of between 4 - 96% in predominately but exclusively running events (ter Steege et al., 2008; de Oliveira et al., 2014; Haaf et al., 2014; Costa, et al., 2017). Symptom severity reported varied from minor (i.e. wind, bloating) to severe/critical (cramps, diarrhoea, acute colitis, faecal occult blood, chronic ischemia) the latter symptoms being frequently expressed as a function of distance covered, intensity and modality particularly in ultra-endurance events (Riddoch and Trinick, 1988; Jeukendrup et al., 2000; Choi et al., 2006; Cohen et al., 2009; de Olivera et al., 2014; Costa, et al., 2016; Roberts et al., 2016; Stuempfle and Hoffman, 2015; Stuempfle et al., 2016).

Similarly it is established that, acute bouts of steady state exercise of varying durations and intensity usually greater than 70% of $\dot{V}O_2$ max performed under a range of environmental conditions also lead to an increased expression of gastrointestinal symptoms and detection of biomarkers, indicative of small and large intestinal permeability and tissue damage (Pals, et al., 1997; Jeukendrup et al., 2000; Marchbank et al., 2011; van Wijck et al., 2011; van Wjick et al., 2013; Davidson et al., 2016; Costa et al., 2017; Pugh et al., 2017; Snipe et al., 2017; Chantler et al., 2021). The exact physiological mechanisms of these gastrointestinal biomarker changes remain to be fully clarified, although contributory mechanisms include exercise intensity and duration dependent changes in: splanchnic blood flow/perfusion (van Nieuwenhoven, 1999; Otte et al., 2001; Rowell, 2004; ter Steege et al., 2008; van Wijck et al., 2011), splanchnic hyperthermia (Lambert et al., 2002b; Dokladny et al., 2006; Zhul et al., 2014; Pires et al., 2016; Snipe et al., 2017), GI mucosal tissue hypoxia and disruption to enterocyte tight junction proteins (van Wjick et al., 2011; Dokladny et al., 2016; Zuhl et al., 2014) and mucosal oxidative

stress and inflammation (Deibert et al., 2005; Fehrenbach and Schneider 2006; Lim and Mackinnon, 2006; Selkirk et al., 2008).

Taken together it is clear that both the subjective expression of gastrointestinal symptoms and objective biomarkers respond adversely to exercise when exceeding critical thresholds of activity duration or intensity (Pals et al., 1998; Lambert et al., 2000; Costa et al., 2017; Chantler et al., 2021). However, these subjective and objective markers whilst responding to steady state exercise of different intensities and durations have not been adequately explored during other forms of exercise activity. Due to many sports involving frequent bursts of high intensity movements, athletes are increasingly using high intensity training to improve exercise performance (Bishop and Turner, 2017). High intensity interval training (HIIT), or sprint interval training (SIT/RSA) is a form of intermittent training that alternates short periods of intense anaerobic exercise with less intense active or passive recovery periods over multiple repetition and sets (Gibala and Jones, 2013; Gibala et al., 2021). HIIT is performed to provide athletes enhanced $\dot{V}O_2$ max capacity and improved metabolism (Reljic, et al., 2018; Gibala et al., 2021). Whilst the physiological and metabolic responses to intermittent exercise are well profiled, limited consideration of gastrointestinal symptomology, permeability and damage occurring because of undertaking high intensity intermittent and repeated sprint exercise remains sparse but may provide an effective damaging protocol (Pugh et al., 2017; Hamzah et al., 2018; Wallet et al., 2021).

Since strenuous exercise may disrupt enterocyte function, consequently it may also impair macronutrient absorptive capacities of the gastrointestinal tract which has implications for the implementation and effectiveness of nutrient replenishment and post exercise recovery strategies (Burke et al., 2019). Several studies have noted impairments in the absorption rate of a deuterium tracer across small intestine during exercise and observed impairments become more pronounced as exercise intensity increased (Maughan et al., 1990; Lambert et al., 1999). More recently, van Wjick et al. (2013) noted impairment in protein digestion and absorption that correlated with biomarkers of gut damage following a period of acute resistance exercise (van Wjick et al., 2013). In animal and human clinical models' reductions in small intestine nutrient absorption is commonly noted under high stress conditions (Jha et al., 2019; Cui et al., 2022). Contrary to these findings Costa et al. (2020), observed no impairment in a whey-based protein absorption relative to control condition despite the presence of gastrointestinal biomarkers of gut damage. Appropriate nutritional interventions may be required to promote

maintenance of gut function/barrier integrity in such scenarios to ameliorate potential adverse effects on enterocyte function because of exercise activity.

Nucleotides serve as a nutritional pathway to support the structural and functional integrity of the gastrointestinal tract. To maintain this structural and functional integrity the enterocytes of the gastrointestinal system which form the epithelial layers of the mucosa are rapidly dividing tissues, with high rates of proliferation and growth to counteract the loss of cells in the proximal villus due to physical, microbiota, oxidative or chemical stress (Wong and Wright 1999; Grimble and Westwood, 2001; Turner, 2009; Grootjans et al., 2016; Camilleri and Velds 2021). In humans, the GI mucosa undergoes full cellular renewal every ~72-96 hours (Dancey et al., 2006; McCole and Barrett, 2007; Jaladanki and Wang, 2016; Chung et al., 2021). This high turnover rate requires substantial availability of nucleic acids. However, enterocytes have limited capacity for de novo synthesis of purine and pyrimidine nucleotides and rely largely on nucleotide salvage pathways to maintain gastrointestinal barrier proliferation and function (Savaiano et al., 1981; Bissonnette, 1992; Walker, 2006; Hess and Greenberg, 2012).

In healthy adults, dietary intake and endogenous nucleotide production and salvage generally meets nucleotide requirements, rendering supplementation unnecessary. However animal and human studies indicate that adding exogenous nucleotides to animal feed and infant formula support the maintenance, repair, and enhancement of gastrointestinal barrier both morphologically and functionally when 'physiological stress' levels are high as well as alleviating subjective gastrointestinal symptoms (Gil, 2001; Carver, 2003; Dancey et al., 2006; Hess and Greenberg, 2012; Che et al., 2016; Ding et al., 2021; Valini et al., 2021). Exercise stress may also compromise gastrointestinal barrier integrity, impacting both structure and function (Lambert et al., 2001) potentially transitioning exogenous dietary nucleotides from being a non-essential to conditionally essential status in rapidly dividing tissue of the gut (Van Buren and Rudolph 1997; Sanchez-Pozo and Gil, 2002; Hess and Greenberg, 2012; Xu et al., 2017; Ding et al., 2021). Whether such a scenario exists in the gastrointestinal system of athletes whose macro and micronutrient requirements are elevated to meet training and recovery needs is unknown. Given protein and whey isolates are low in or do not report nucleotide concentration and nutritional analysis of athletes' diets exclude nucleotide concentration estimates there may exist a deficit in the purine and pyrimidine nucleotides in the athletic diet. The possibility that exogenous nucleotide supplementation could enhance bioavailability for gastrointestinal tissues, thereby preserving gut barrier integrity, function and

reducing gastrointestinal symptoms, especially during acute exercise-induced stress, has not been widely investigated. This study aims to examine the effects of chronic exogenous purine and pyrimidine nucleotide ingestion on gastrointestinal permeability, gastrointestinal damage, and gastrointestinal symptom expression following acute high-intensity repeated sprint exercise. It is hypothesised that nucleotide supplementation will attenuate the increase in these biomarkers compared to control and placebo conditions.

3.3 Methods

3.3.1 Participants Characteristics

Fifteen recreationally active male participants (Table 3.1.0.) volunteered to participate and undertook a double-blind repeated measures design (Figure 3.1.1 and 3.1.2).

Table 3.1.0. Descriptive characteristics of participants displaying means $\pm$ SD for age (years); body mass (kg); height (cm) and $\dot{V}O_{2\text{ max}}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$).

Variables	Participants ($n=15$)
Age (years)	21.7 ± 4.2
Body Mass (kg)	72.4 ± 6.7
Height (cm)	177.7 ± 5.0
$\dot{V}O_{2\text{ max}}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	49.0 ± 5.1

3.3.2 Experimental Protocols and Designs

Participants were required to attend the lab on 4 separate visits of varying duration for an initial $\dot{V}O_{2\text{ max}}$ fitness assessment via a 20-meter shuttle test (Ramsbottom, 1995) and repeated anaerobic sprint test RAST familiarisation trials. This was then followed by a baseline assessment of RAST performance and baseline measure of gastrointestinal permeability, damage, and subjective gastrointestinal symptomology. Participants were asked to refrain from exercise for 24 hours prior to physiological assessment. All participants then undertook a baseline anthropometry and performance assessment consisting modified repeated anaerobic sprint test consisting of 4 sets of 6 x 35m sprints with a passive recovery period of 5 minute

separating each set. After each sprint, heart rate (HR) (Polar, Kempele, Finland), Ratings of Perceived exertion (RPE) (Borg, 1970; Scale 6-20), Sprint Times (s) (ST) were recorded. Thermal comfort and subjective gastrointestinal symptomology via the Pfeifer scale was determined at the end of each set of 6 sprints. Aural (tympanic) temperature was determined from an in-ear infrared thermometer (Genius Infrared Tympanic Thermometer Model 3000A, Cranlea, UK). Fatigue index (%) was derived following the procedures of Fitzgerald et al. (1993). A 10 mL venous blood samples were drawn to determine plasma/serum biomarkers of gastrointestinal permeability and damage as well as uric acid and cortisol concentrations. A finger prick blood sample (10ul) for Blood Lactate (La) was taken by a safety lancet (Sarstedt, Germany) and analysed by a lactate pro analyser (Lactate Pro, Arkray, Japan) at rest and after each sprint set. Each participant completed a total of 24 sprints (4 sets X 6 sprints sets) per visit. Upon completion of baseline testing the participants were then allocated to a 30-day nucleotide (n=7) or placebo ingestion period (N= 8), ingesting either nucleotide (Nutri-tide; Pro-bio, Daresbury, UK) derived from yeast (*Saccharomyces cerevisiae*) at the designated loading doses of 2520mg or a maltodextrin and binding agents' placebo in anonymised capsules. The dosing regimen consisted of 6 capsules x 3 times per day after meals. Upon completion of the initial experimental supplementation phase participants returned for assessment followed by physiological RAST assessment and repetition of the gastrointestinal biomarkers and gastrointestinal discomfort scales. A 14 day wash out was undertaken and participants recommenced the experimental design with the alternative treatment condition. No supplements were consumed on the day of assessment. Compliance was determined by returned pill count.

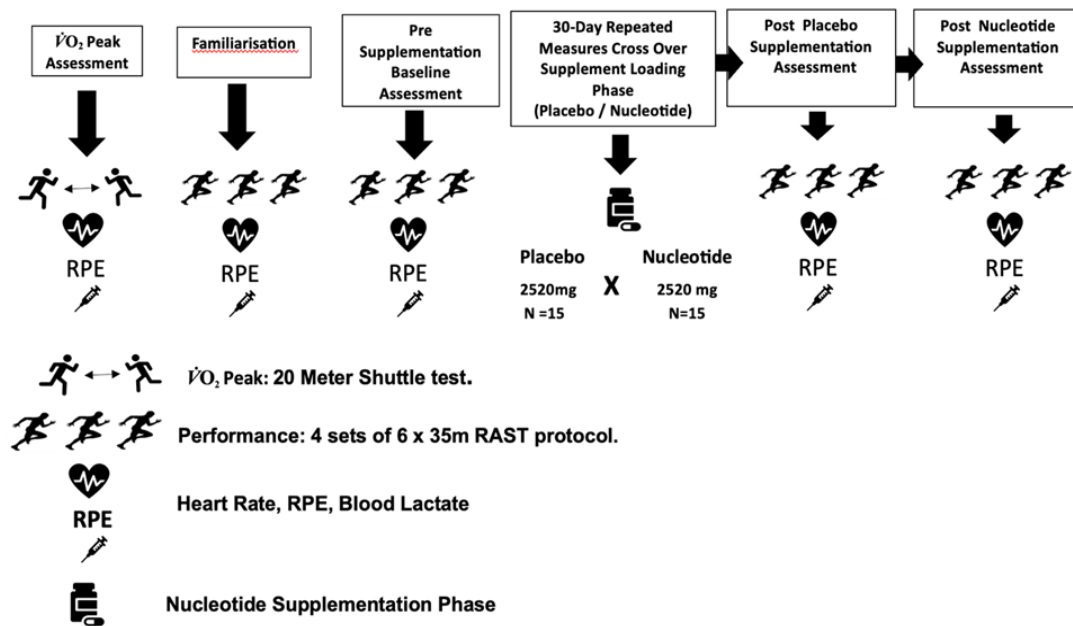


Figure 3.1.1. Experimental design schematic for the double-blind repeated measures nucleotide gastrointestinal trial.

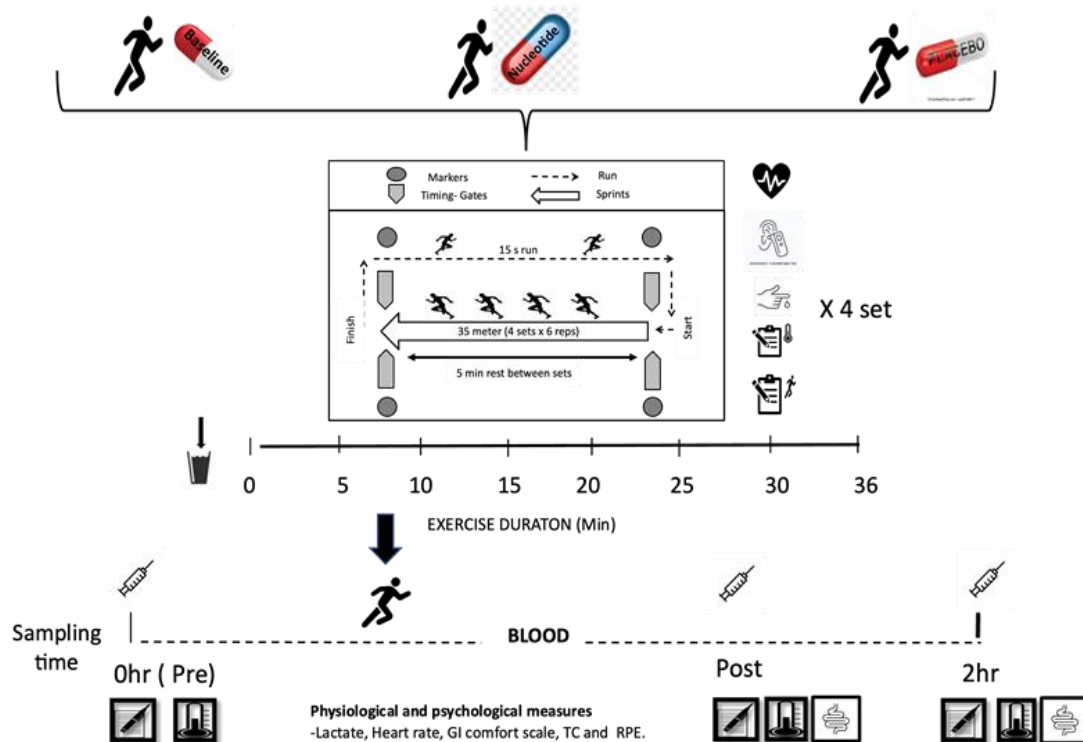


Figure 3.1.2. Experimental performance protocol undertaken under each condition and outcomes.

3.3.3 Anthropometry: Body Mass and Height

Following the provision of informed consent, each participant underwent assessment of anthropometry during the first visit of their respective trials. Using a stadiometer (SECA 213, Cranlea and Co, Bournville, Birmingham, UK), height was measured from the vertex of the cranium to the plantar surface of the foot and recorded in centimetres (cm) to one decimal point. Body mass was also recorded using digital column scales (SECA 704, SECA, UK) and recorded in kilograms (kg) to one decimal point. The procedures followed the guidelines of the International Society for Kinanthropometry guidelines ISAK (Marfell-Jones et al., 2006).

3.3.4 Cardiorespiratory Measurements: Estimated Maximal oxygen uptake (20-meter shuttle test)

All participants completed an incremental exercise 20-meter multistage shuttle (20-MST) run test on the physiology runway of the TRB building for the estimation of maximal oxygen uptake (Ramsbottom et al., 1985). Prior to beginning the test, a Polar HR monitor was fitted and recorded throughout. The 20-MST running test commenced at $8.5 \text{ km} \cdot \text{h}^{-1}$ and increased by $0.5 \text{ km} \cdot \text{h}^{-1}$ every 1 minute with participants running between two lines set 20 m apart at a pace dictated by an audio signal emitted at appropriate intervals to replicate the running speed required. The test was terminated when the participant failed twice to reach within 3 m of the 20-MST line in time with consecutive tones (objective evaluation) or the participant felt unable to complete another shuttle at the audio signal designated speed (subjective evaluation). The total number of levels and shuttles covered during the 20-MST (including the last incomplete shuttle) was designated as the testing score and converted to an estimated aerobic capacity using a conversion formula (Paradisis et al., 2014). Laboratory environmental conditions were recorded during all exercise testing procedures and remained constant with a temperature at $19.4 \pm 3^\circ\text{C}$ and relative humidity at $44 \pm 5\%$. Rating of perceived exhaustion (RPE) were implemented to anchor the perception of effort to be utilised later during the repeated anaerobic sprint test trials (Draper and Whyte, 1997).

3.3.5 Heart Rate

Heart rate (HR) ($\text{b} \cdot \text{min}^{-1}$) was continually monitored and recorded during all experimental trials by a wearable electrocardiography (Polar FT1, Polar UK). In all studies, heart rate ($\text{b} \cdot \text{min}^{-1}$) was continually measured at 5-second intervals; data as presented represents an average over designated time periods. The polar HR chest strap to determine heart electrical activity was worn directly below the chest at $\sim\text{V5}$ level with the transmitter positioned centrally on the

xipho-sternum then adjusted to minimise any movement during each exercise trial. ECG gel was applied to the electrodes to increase conductivity of the signal and improve reliability of measurement in the early stages of exercise.

3.3.6 Rating of Perceived Exertion (RPE)

RPE was recorded, using the Borg 6-20 (Borg, 1970) scale, at the end of each shuttle stage during the multi-stage maximal oxygen uptake assessment and at the end of each set within the RAST experimental tests. Participants were familiarised using the Borg scale during the initial maximal incremental exercise test to exhaustion. Each end of the Borg scale was ‘anchored’ during the maximal exercise protocol to introduce the perception of effort associated with progression through the scale from 6 [rest] through 20 [maximal effort], (Birk and Birk, 1987). This process provides a reference point against which participants can refer/gauge their subsequent efforts during exercise trials.

Rating	Description
6	No exertion at all
7	Extremely Light
8	
9	
10	Very Light
11	
12	
13	Somewhat Hard
14	
15	
16	Hard
17	
18	
19	Very Hard
20	
	Extremely Hard
	Maximal Exertion

Borg scale for reporting subjective rating of Perceived exertion (Borg, 1970).

3.3.7 Thermal Comfort

Subjective rating of thermal comfort was obtained at the same time-points as heart rate using a 9-point VAS, adapted from Snipe et al. (2018a).

Thermal Comfort Scale	
1	Very Cold
2	Cold
3	Cool
4	Slightly Cool
5	Neutral
6	Slightly Warm
7	Warm
8	Hot
9	Very Hot

Subjective assessment of participants thermal comfort scale as an indicator of perceived thermal strain.

3.3.8 Tympanic Aural Temperature (°C): Core Temperature

Aural temperature as a representation of core temperature was also obtained. Aural temperature was collected using a digital thermometer (Genius Infrared Tympanic Thermometer Model 3000A, Cranlea, UK) according to the manufacturer's instructions. The Genius 3000A thermometer is designed to read the infrared energy emitted by the tympanic membrane and surrounding tissues to estimate the person's core temperature.

3.3.9 Collection, Preparation, Storage and Analysis of Blood Samples

Venous blood samples were collected by a qualified phlebotomist from a superficial vein in the ante-cubital fossa of the forearm using standard venepuncture techniques (Vacutainer Systems, Becton, Dickinson). Briefly, a tourniquet was applied to the upper arm to occlude venous return; the median antecubital vein was cleaned with a Medi-swab isopropyl alcohol

wipe. The butterfly needle (BD safety set 21 G $\frac{3}{4}$ Becton Dickinson, Oxford, UK) was inserted, once a flashback was observed, the needle secured, and the tubes were attached. Upon completion of the sampling the needle was removed, and pressure applied to the puncture site to minimise the potential for a subcutaneous haematoma. All blood samples were collected into vacutainers™ of differing volumes (6.0 mL – 10 mL) (Becton Dickinson, Northampton UK). The blood was sampled using a winged infusion set (butterfly needle; Becton Dickinson, Northampton UK). The samples were collected into four vacutainers; a serum separation tube, EDTA and lithium heparin which was used as a clotting or chelating agent for the blood. Briefly the serum separation tube was allowed to sit at room temperature for approx. 30 minutes until clotted then placed on ice until centrifugation. The samples were then centrifuged (Sigma 3-18 k Scientific Laboratory Supplies, UK) at 1300 x g for 10 minutes at 4°C (Serum and EDTA) or at 1000 X g for 10 minutes at 4°C. The plasma/serum was then carefully removed from the vacutainer using a pipette (Fisherbrand, Finnpiptette, U.S.A.) and aliquoted into replicates. Following this, plasma and serum were then stored at -80°C (Thermo, ULT Freezer, Ohio U.S.A.).

3.3.10 Capillary Blood Lactate Collection and Analysis

Capillary blood lactate was determined from a finger puncture test. Briefly at the end of each exercise set a finger (1st- 3rd digit) was cleaned with an alcohol swab to remove any sweat or blood and then the skin was penetrated with a sterile single-use lancet (Sarstedt, Germany). Approximately 10 µL of blood was withdrawn via gentle squeezing action upon the finger. Test strips were inserted into a Lactate pro (Arkray, Kyoto, Japan) device which were then placed against the surface of this blood sample until beeps were audible, signifying the start of analysis. The broad principle of the assay is presented in chapter 2, section 2.3.8. The same investigator collected and analysed all blood samples throughout the study.

3.3.11 Intestinal Permeability (Dual Sugar Probe Administration)

To determine GI permeability, serum samples drawn from participants underwent a gastrointestinal permeability assay (Fleming et al., 1996). Each participant ingested a solution containing a mixture of a mono- and disaccharide sugar probes. The probes consisted of 5g lactulose and 1g L-rhamnose, diluted in 150 mL of tap water (Fleming et al., 1996). Ingestion of the probe solution occurred ~15 minutes prior to exercise commencement to allow for gastric emptying in line with recommendations (Fleming et al., 1996). Figure 3.1.2 demonstrates a schematic representation of the study. Analysis of sugar probe permeability was determined

from serum collection, immediately post and two hours post-ingestion of the sugar probe solution. High-performance liquid chromatography (HPLC) was used to determine serum sugar concentrations using a previously published protocol (Fleming et al., 1996), with the modification of using L-rhamnose instead of mannitol as the monosaccharide probe.

Eluent preparation. Deionised water (968 mL, 18 Mohm cm^{-1}) was degassed with helium for 20 min, 3 mL of 0.5 mol/L zinc acetate was added and the solution was thoroughly mixed and sparged for 5 min. To this was added 28 mL of 500 g/L sodium hydroxide with thorough mixing and sparging for a further 5 min to give a final eluent composition of $360 \text{ mmol}\cdot\text{L}^{-1}$ NaOH and $1.5 \text{ mmol}\cdot\text{L}^{-1}$ zinc acetate. A second eluent of deionised water was sparged for 20 min with helium. Both eluents were stored under helium at 34.5 kPa. A final eluent concentration of $120 \text{ mmol}\cdot\text{L}^{-1}$ NaOH and $0.5 \text{ mmol}\cdot\text{L}^{-1}$ zinc acetate was achieved by mixing eluent 1 with eluent 2 in proportions of 33.3:66.7 (by vol) on the chromatographic system. The sample preparation required that a 200- μL volume of serum was added to 200 μL of internal standard (melibiose $250 \text{ mg}\cdot\text{L}^{-1}$). To this, 200 μL of ice-cold 5-sulfosalicylic acid ($35 \text{ g}\cdot\text{L}^{-1}$) was added to precipitate the plasma proteins. After standing on ice for 20 min, the samples were centrifuged at 900g for 5 min and the supernates were removed. Each supernate was desalted with the mixed ion-exchange resin and centrifuged again; 25 μL of this supernate was then injected onto the column. The samples were eluted with the NaOH: zinc acetate eluent at a flow rate of 1 mL min at 25°C . After every five serum samples, the column was given a 5-min wash with 1 mol/L NaOH to maintain the stability of the retention times. The various sugars were separated using high-performance liquid chromatography (HPLC) and quantitated by use of a pulsed electrochemical detector using a gold working electrode and silver/silver chloride reference electrode. The detection potential was -0.01 V (0-0.5 s), the oxidation potential was $+0.75 \text{ V}$ (0.51-0.64 s), the reduction potential was -0.75 V (0.65-0.75 s), and the integration period was 0.05 to 0.5 s. Retention times were 2.7 min for L-rhamnose and 6.1 min for lactulose. The coefficient of variation for the samples in the trial using this method has been found to be between 1.8 – 8.5 % (Fleming, personal communications, 2018). Quantification of lactulose, L-rhamnose, and the ratio of lactulose to L-rhamnose was used to evaluate small intestinal permeability.

3.3.12 Intestinal Fatty Acid Binding Protein (I-FABP)

I-FABP was determined by analysis of plasma samples using an inhouse Duo Set ELISA kit (R and D systems, Abingdon, Oxford, UK) with a detection range of 31.5-2000 pg·mL⁻¹ according to the manufacturer's instructions. I-FABP concentrations (pg·mL⁻¹) were measured in samples taken pre-exercise, immediately post-and 2 hrs post exercise. Briefly, as an in-house test the plate preparation was required 24 hours prior to use. Briefly, IFABP capture antibody was diluted to the working concentration in phosphate buffered saline. Immediately the solution was used to coat a 96-well microplate with 100 µL per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature in the dark. Following overnight incubation, the assay procedure was started with each well emptied by inverting and blotting the contents on a clean paper towel with gentle tapping on the bench top. It was then washed with the wash buffer (400 µL) per well, repeating this 'wash' process for total of three washes. After the last wash, remaining wash buffer was removed by inverting and tapping the plate and blotting it against clean paper towels. Each plate was then blocked by adding 300 µL of the reagent diluent to each well and incubated at room temperature for one hour. Upon completion, wells were emptied and washed as outlined. Standards, controls, and samples (100 µL) were added to the plate per well. The plate was covered and incubated 2 hours at room temperature. Upon completion, the emptying and wash step was repeated and 100 µL of detection antibody added to each well and the plate covered and incubated for a further 2 hours at room temperature. The wash step was repeated, followed by the addition of 100 µL of Streptavidin-HRP A to each well. The plate was covered again and incubated for 20 minutes at room temperature with the washing repeated followed by the addition of 100 µL of substrate solution to each well. It was then incubated for 20 minutes at room temperature. Following this 50 µL of stop solution was added to each well with gentle tapping to mix. The optical density of the solution was determined immediately using a microplate reader (Tecan Microplate, Oxford, UK) set to 450 nm with wavelength correction set to 540 nm. This subtraction acted to correct for optical imperfections in the plates. Samples were run in duplicate. The intra-assay coefficient of variation for the control samples assayed on each plate was ~17 %.

3.3.13 Gastrointestinal General Symptomology

An assessment of subjective gastrointestinal symptoms was performed using a 10-point Likert questionnaire at time-points to coincide with those of blood sample collection for GI permeability and damage assessment. Participants completed a questionnaire detailing a range

of symptoms with a visual analogue scale (VAS; 100 mm lines) (Pfeiffer et al., 2009; Pugh et al., 2017). Participants were also asked to indicate by marking the scale, indicating the magnitude of different symptoms, with 0 indicating no symptoms to 10 indicating severe symptoms. These GI symptoms were reported in terms of percentage, with 0% indicating no symptoms, and 100% representing severe symptoms) with a score ≥ 4 being regarded as indicating GI distress (Pfeiffer et al., 2009). Symptoms of gastrointestinal discomfort included on the questionnaire: side stitch; nausea; bloating; urge to burp; urge to vomit; urge to defecate; diarrhoea; stomach cramps; stomach upset; intestinal cramps; dizziness; shivering; and heart burn.

3.3.14 Repeated Anaerobic Sprint Test Exercise Protocol

A high intensity intermittent exercise sprint protocol was performed by each participant. The protocol a modified version of the Draper and Whyte (1997) Repeated Anaerobic Sprint Test (RAST) consisted of 4 sets of 6 x 35 m sprints, with 15 second active run recovery to the commencement start after each sprint and a 5-minute passive recovery separating each set (Figure 3.1.3). Briefly, the test commenced with the participant undertaking a maximal exertion 35-meter sprint with participant then running back (sub-maximal recovery) to the start line before commencing the next sprint with a time limit of 15 seconds between each sprint. Each sprint was automatically timed (Brower Timing System, USA), and HR, and RPE (Borg Scale 6-20), were recorded after each sprint. Blood lactate was recorded prior to exercise commencement (baseline) after each set of 6 X 35m sprints and 5 minutes post protocol completion. Fatigue index (FI) was determined using the percentage decrement calculation (Fitzsimmons et al., 1993):

$$\text{Fatigue} = (100 \times [\text{total sprint time} / \text{ideal sprint time}]) - 100$$

Total sprint time= sum of sprint time from all sprints.

Ideal time= number of sprint x fastest sprint time.

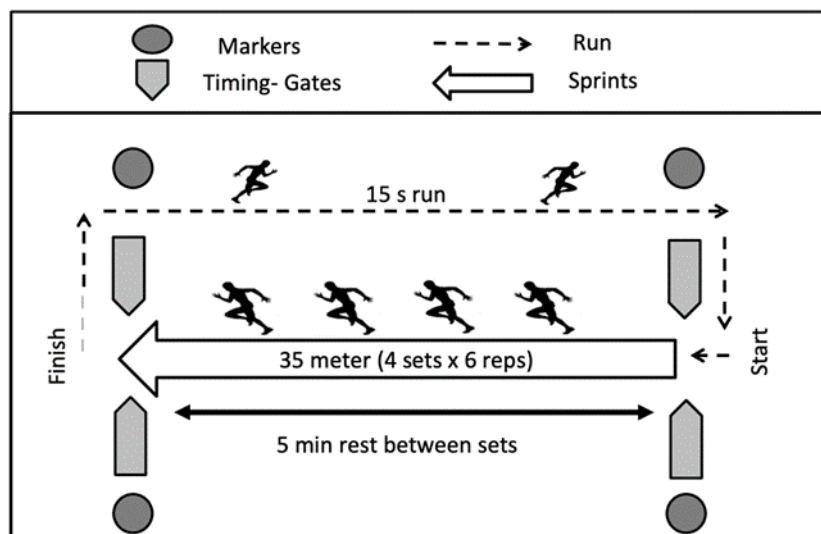


Figure 3.1.3. Protocol schematic for Repeated Anaerobic Sprint Test (Modified Draper and Whyte, 1997).

3.3.15 Dietary Nucleotide Supplementation

Across the studies participants ingested a capsule consisting of an outer gelatine shell with either 5'-ribonucleotides/RNA nucleotides (Nutri-tide; Pro-bio, Daresbury, UK) derived from yeast (*Saccharomyces cerevisiae*) at the designated loading doses of 2520 mg or a maltodextrin (250 mg) and binding agents' placebo in anonymised capsules. The nucleotide mix was a proprietary mix of purine and pyrimidine nucleotides including the following containing cytosine, uridine, thymidine, adenosine, and guanine.

3.3.16 Statistical Analysis

Statistical analysis was carried out using the Statistical package for Social Science software program (SPSS V. 23, for Windows, Chicago, Illinois, USA). All Data was presented as means ($\pm$ SD). Data was assessed for normality of distribution. Two-way within subject ANOVA 'condition' and 'time' was applied to the above parameters. To correct any violations of sphericity the degrees of freedom were corrected using Greenhouse-Geiser epsilon (<0.75) or Huynh-Feldt epsilon (≥ 0.75), as applicable (Field, 2000). Post-hoc Tukey analysis was used to detect difference where a main effect was apparent. Statistical significance was set at $p \leq 0.05$ for all tests. To assess data on GI symptoms, a descriptive approach was used, as GI symptom scores were mainly described at the lower end of the detection scales utilised.

3.4 Results

3.4.1 Repeated Anaerobic Sprint Test Performance

All participants successfully completed the 4 sets of 6 x 35m high intensity intermittent exercise sprint protocol before supplementation, after 30-days of nucleotide, washout, and placebo supplementation. Average sprint times across sets pre and post supplementation are presented (Table 3.1.1).

Table 3.1.1. Sprint performance output of different supplement condition. No significant effects of dose pre and post supplementation were evident. Data are expressed as Mean $\pm$ SD.

Supplement Condition	Sprint Time (s)				P value
	Set 1	Set 2	Set 3	Set 4	
Baseline	5.94 $\pm$.054	6.12 $\pm$ 0.46	6.30 $\pm$ 0.50	6.30 $\pm$ 0.52	$p \geq 0.05$
Post Placebo	6.01 $\pm$ 0.44	6.22 $\pm$ 0.56	6.41 $\pm$ 0.59	6.55 $\pm$ 0.70	$p \geq 0.05$
Post Nucleotide	5.93 $\pm$ 0.48	6.07 $\pm$ 0.47	6.30 $\pm$ 0.51	6.46 $\pm$ 0.76	$p \geq 0.05$

During pre-supplementation phase, the mean sprint performance for all 4 sets combined, were 6.17 $\pm$ 0.47s while post 30-days placebo loading were 6.30 $\pm$ 0.56s and the post 30-days nucleotide loading were 6.19 $\pm$ 0.52s (Figure 3.1.4). There were no significant main effects or interaction effects of 30-days nucleotide supplementation upon sprint performance ($F_{3.1, 43.7} = 1.27$, $P = 0.30$).

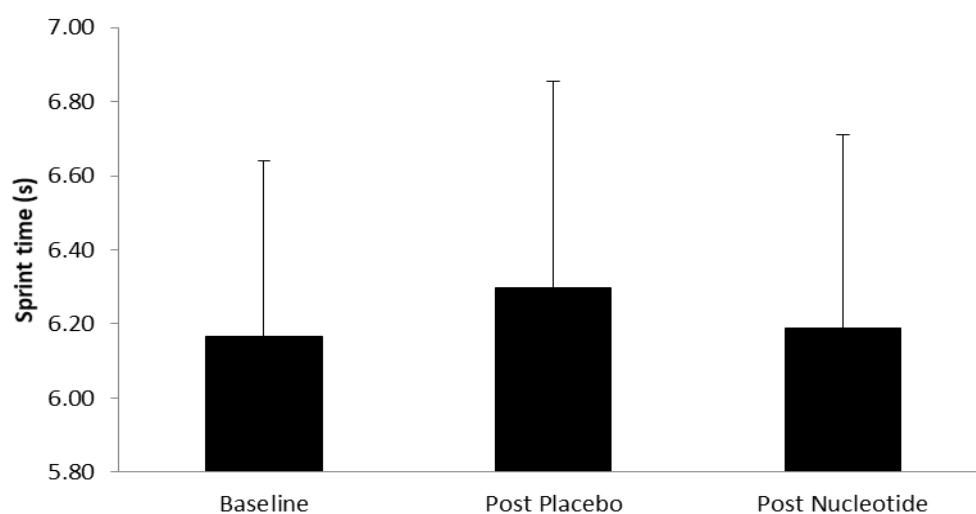


Figure 3.1.4. Average sprint time (s) for all participant's over 24 sprints at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

3.4.2 Fatigue Index Data

During pre-supplementation phase, the mean fatigue index (%) for all 4-sets combined, were 20.92 ± 8.96 while post 30-days placebo loading were 17.96 ± 5.86 and the post 30-days nucleotide loading were 20.96 ± 12.52 . There was no significant main effect or interaction effects of 30-days nucleotide supplementation upon fatigue index pre and post supplementation ($F_{1,8,25.1} = 1.11$, $P = 0.34$).

Table 3.1.2. Average fatigue index responses(s) for all participants across all sets at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Supplement Condition	Fatigue Index (%)				P value
	Set 1	Set 2	Set 3	Set 4	
Baseline	$11.6 \pm .6.3$	$14.5 \pm 0.7.7$	12.6 ± 5.7	10.6 ± 4.1	$p \geq 0.05$
Post Placebo	11.28 ± 4.8	12.6 ± 6.0	12.6 ± 6.8	10.3 ± 7.4	$p \geq 0.05$
Post Nucleotide	10.3 ± 6.3	14.4 ± 9.4	13.9 ± 10.9	13.4 ± 11.5	$p \geq 0.05$

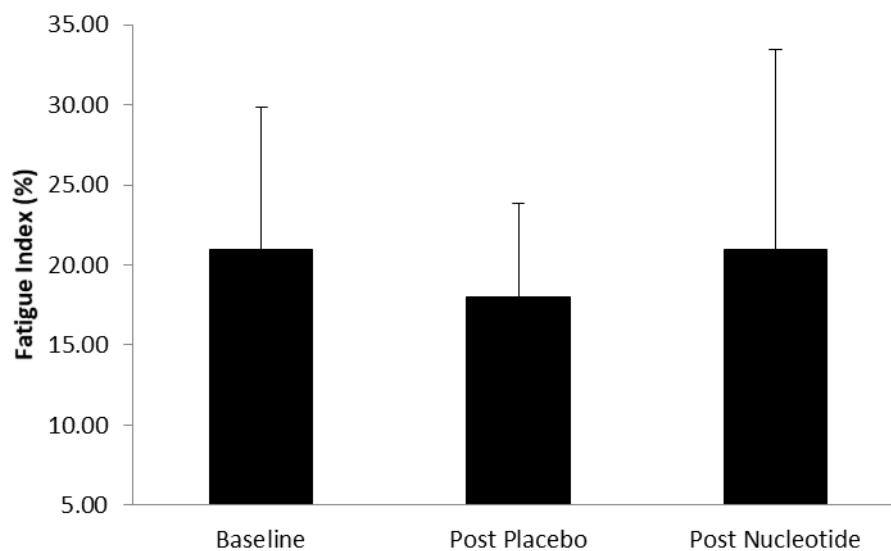


Figure 3.1.5. Average fatigue index (%) responses(s) for all participants across all 24 sprints at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

3.4.3 Heart Rate Data

During pre-supplementation phase, the mean heart rate for all 4-set combined, were 167 ± 12 $\text{b} \cdot \text{min}^{-1}$ while post 30-days placebo loading were 169 ± 11 $\text{b} \cdot \text{min}^{-1}$ and the post 30-days nucleotide loading were 168 ± 10 $\text{b} \cdot \text{min}^{-1}$. There was no significant main effect or interaction effect of 30-days nucleotide supplementation upon heart rate responses during sprint performance pre and post supplementation ($F_{3.0, 42.0} = 0.34$, $P = 0.79$).

Table 3.1.3. Average heart rate responses(s) ($\text{b} \cdot \text{min}^{-1}$) for all participants across all sets at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Supplement	Heart Rate ($\text{b} \cdot \text{min}^{-1}$)				
Condition	Set 1	Set 2	Set 3	Set 4	<i>P</i> value
Baseline	160 ± 14	167 ± 13	169 ± 12	171 ± 11	$p \geq 0.05$
Post Placebo	163 ± 11	169 ± 11	171 ± 12	171 ± 13	$p \geq 0.05$
Post Nucleotide	163 ± 11	169 ± 11	170 ± 11	172 ± 11	$p \geq 0.05$

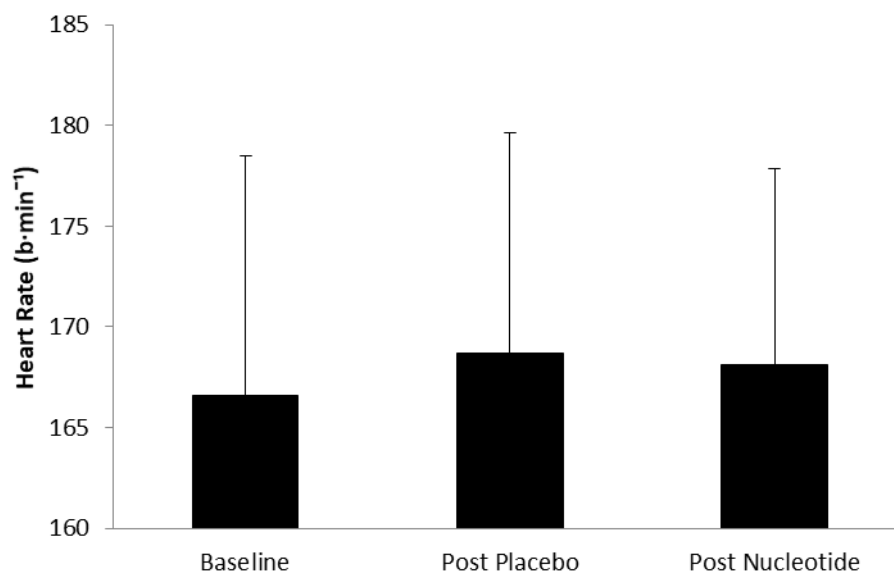


Figure 3.1.6. Average heart rate ($\text{b} \cdot \text{min}^{-1}$) for all 24 sprints combined. Data are expressed as Mean $\pm$ SD.

3.4.4 Rating of Perceived Exertion (RPE) Data

During pre-supplementation phase, the mean RPE for all 4-sets of repeated sprint activity combined, were 15 ± 2 while post 30-days placebo loading were 15 ± 1 and the post 30-days nucleotide loading were 15 ± 1 . There was no significant main effect or interaction effect of 30-days nucleotide supplementation upon the rating of perceived and exertion during each sprint performance pre and post placebo or nucleotide supplementation ($F_{3.7, 51.6} = 0.77$, $P = 0.54$).

Table 3.1.4. Average RPE responses(s) (AU) for all participants across all sets at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Supplement Condition	RPE				P value
	Set 1	Set 2	Set 3	Set 4	
Baseline	13 ± 2	15 ± 2	16 ± 2	16 ± 2	$p \geq 0.05$
Post Placebo	12 ± 2	15 ± 1	16 ± 2	16 ± 2	$p \geq 0.05$
Post Nucleotide	12 ± 2	15 ± 2	16 ± 2	16 ± 1	$p \geq 0.05$

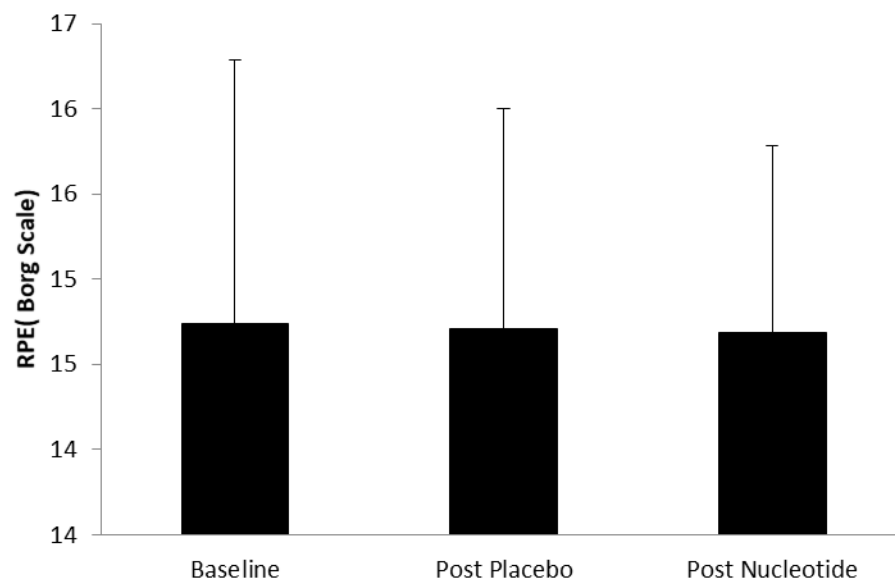


Figure 3.1.7. Average RPE (Borg, 1970) across all 24 repeated sprints combined. Data are expressed as Mean $\pm$ SD.

3.4.5 Blood Lactate Data

During pre-supplementation phase, the mean blood lactate for all 4-sets combined were $11.5 \pm 2.94 \text{ mmol}\cdot\text{L}^{-1}$ while post 30-days placebo loading were 10.4 ± 1.9 and the post 30-days nucleotide loading were 10.30 ± 1.8 . There was no significant main or interaction effect of 30-days nucleotide supplementation on blood lactate responses during each set of sprint performance pre and post supplementation ($F_{2.0, 27.5} = 2.16$, $P = 0.14$).

Table 3.1.5. Average blood lactate concentration ($\text{mmol}\cdot\text{L}^{-1}$) for all participants across all sets at baseline, after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Supplement Condition	Blood lactate ($\text{mmol}\cdot\text{L}^{-1}$)				P value
	Set 1	Set 2	Set 3	Set 4	
Baseline	9.9 ± 4.4	11.92 ± 3.1	11.8 ± 2.9	12.1 ± 3.2	$p \geq 0.05$
Post Placebo	10.1 ± 2.1	10.5 ± 2.5	11.6 ± 2.0	11.8 ± 2.0	$p \geq 0.05$
Post Nucleotide	9.7 ± 2.3	10.3 ± 4.9	10.6 ± 3.2	10.9 ± 2.7	$p \geq 0.05$

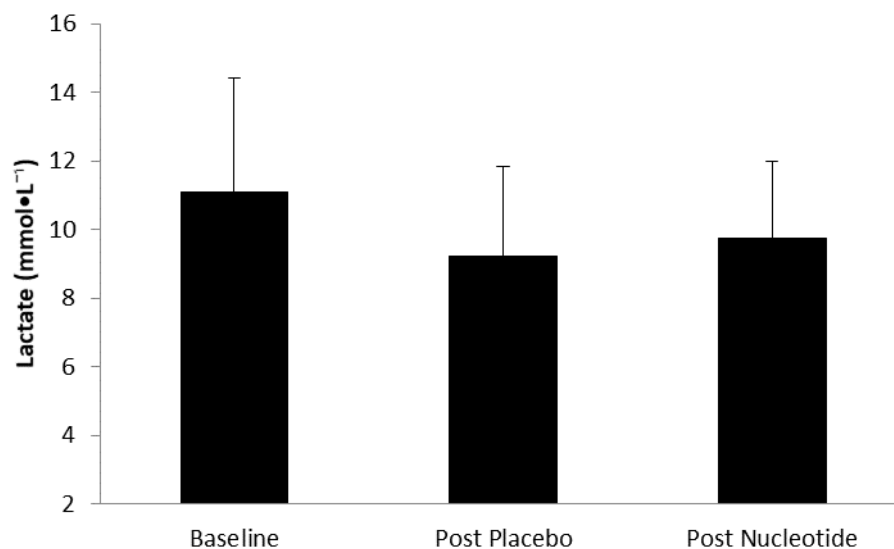


Figure 3.1.8. Blood lactate concentration ($\text{mmol}\cdot\text{L}^{-1}$) post exercise set completion averaged over all 4 sets. Data are expressed as Mean $\pm$ SD.

3.4.6 Thermal Comfort Scale (AU)

The average response to the thermal comfort scale after each sprint set was not significantly different between experimental conditions ($P \geq 0.05$). Thermal Comfort demonstrated an effect of time increasing from set 1 to set 4 in a similar manner across conditions ($P \leq 0.001$).

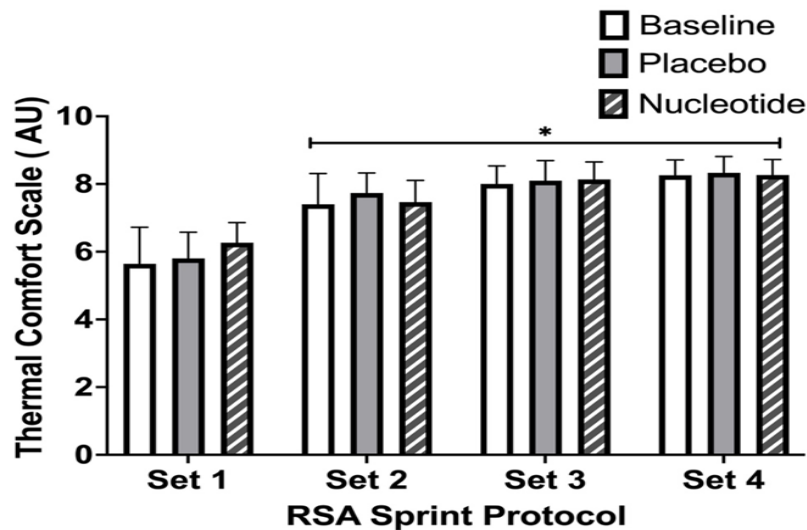


Figure 3.1.9. Thermal Comfort subjective ratings responses during RSA activity prior to (baseline) and following 30 days nucleotide or placebo supplementation. Data are expressed as Mean $\pm$ SD * ($P \leq 0.05$) relative to Set 1.

3.4.7 Tympanic Aural Temperature ($^{\circ}\text{C}$)

There was no significant main effect of experimental treatment ($P \geq 0.05$) (Figure 3.2.0) on estimated core temperature ($P \geq 0.05$). Temperature increased as a function of set repetition from set 1 – 4 ($P \geq 0.05$).

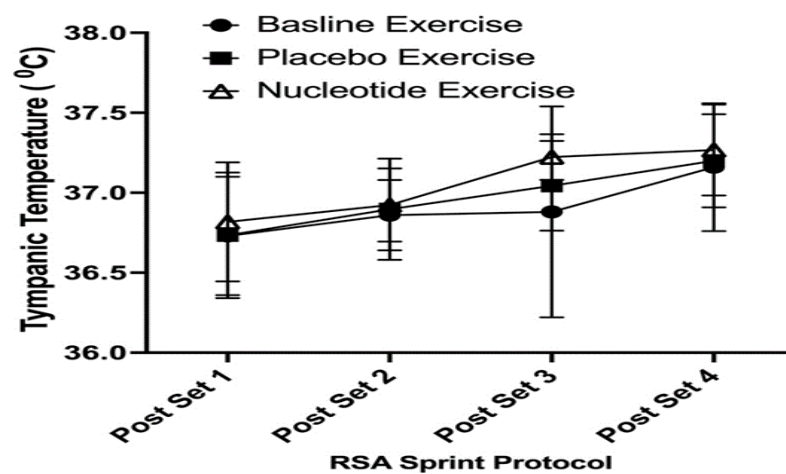


Figure 3.2.0. Tympanic aural temperature ($^{\circ}\text{C}$) during RSA activity prior to (baseline) and following 30 days nucleotide or placebo supplementation measured immediately upon completion of each 6x35 m sprint set. Data are expressed as Mean $\pm$ SD.

3.4.8 Lactulose to L/Rhamnose Ratio

The ratio of serum lactulose to L-Rhamnose (L/R ratio) as a biomarker of gastrointestinal permeability showed no significant different main effect for condition ($F_{1,2} = 6.92$, $P=0.119$). Overall, the L/R ratio concentration was not different between baseline (0.012 ± 0.01), placebo (0.018 ± 0.01) and nucleotide (0.029 ± 0.02) loaded conditions following high intensity RSA exercise 2 hours post ($P \geq 0.05$) (Figure 3.2.1).

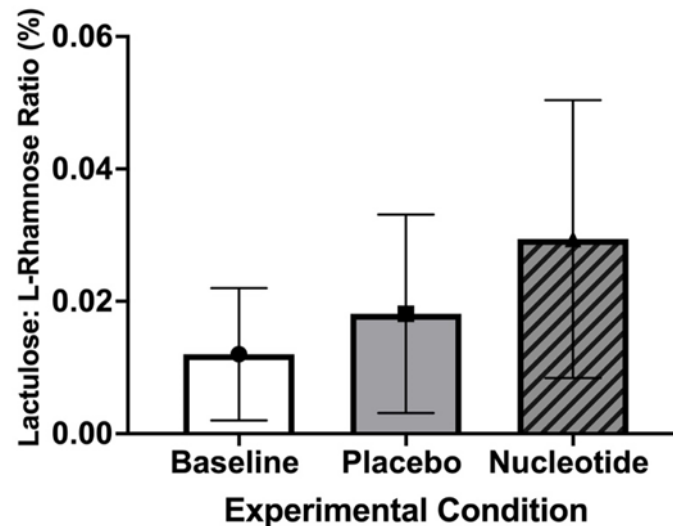


Figure 3.2.1. Lactulose/L-Rhamnose ratio (%) response to RSA activity prior to (baseline) and following 30 days nucleotide or placebo supplementation data are expressed as responses 2 hrs post drink ingestion. Data are Mean $\pm$ SD. No significant difference was determined ($P \geq 0.05$).

3.4.9 Intestinal Fatty Acid Binding Protein (I-FABP) ($\text{pg}\cdot\text{mL}^{-1}$)

Serum Intestinal fatty acid binding protein concentrations are presented in Figure 3.2.2. The mean serum IFABP concentrations were not significantly different between the three treatment conditions of baseline, placebo and nucleotide respectively pre ($468 \pm 151 \text{ pg}\cdot\text{mL}^{-1}$ vs $376 \pm 70 \text{ pg}\cdot\text{mL}^{-1}$ vs $440.6 \pm 104 \text{ pg}\cdot\text{mL}^{-1}$); post ($725 \pm 151 \text{ pg}\cdot\text{mL}^{-1}$ vs $749 \pm 234 \text{ pg}\cdot\text{mL}^{-1}$ vs $822 \pm 321 \text{ pg}\cdot\text{mL}^{-1}$) or 2-hrs post ($404 \pm 128 \text{ pg}\cdot\text{mL}^{-1}$ vs $391 \pm 144 \text{ pg}\cdot\text{mL}^{-1}$ vs $414 \pm 157 \text{ pg}\cdot\text{mL}^{-1}$) indicating no significant differences in small intestine enterocyte damage expressed across treatments. There was no significant effect of time whereby IFABP increased after exercise before returning toward baseline 2 hours post-test ($P \geq 0.05$).

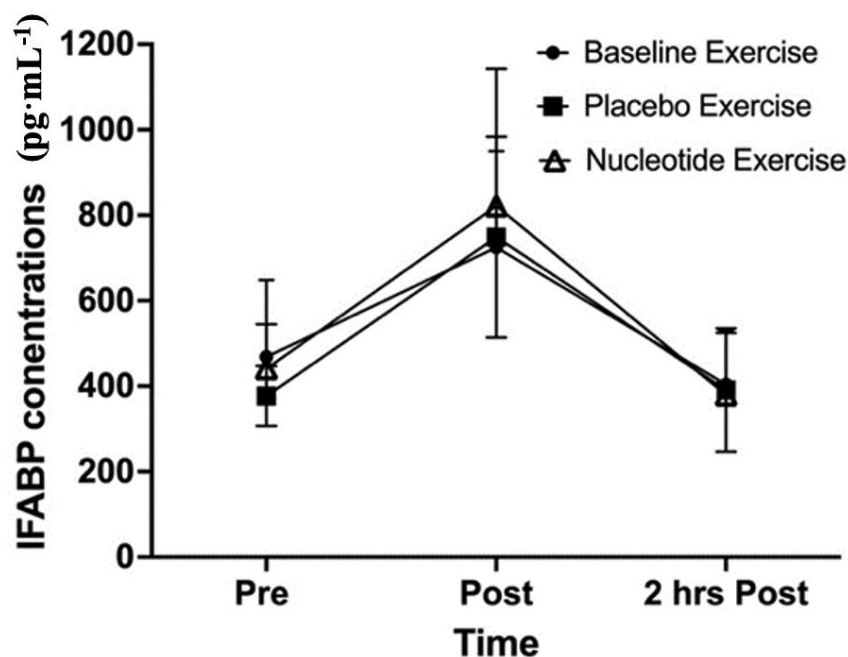


Figure 3.2.2. I-FABP response to RSA activity prior to (baseline) and following 30 days nucleotide or placebo supplementation data are expressed as responses pre, immediately post and 2 hrs post exercise. Data are expressed as Mean $\pm$ SD.

3.4.10 Gastrointestinal Comfort Scale

The average gastrointestinal comfort scale response was not significantly different across baseline placebo or nucleotide supplemented conditions following RSA activity at each time point ($P \geq 0.05$). Several participants displayed signs of GI comfort (≥ 4) immediately following completion of the protocol however these were limited to one to two persons across all conditions. Table 3.1.6 presents the general symptomology expressed by the participants 2 hours after completion of the RSA task no adverse symptoms were determined with the VAS scale approach.

Table 3.1.6. Gastrointestinal symptomology VAS severity rating (mm) expressed pre, post and two hours post undertaking the RSA activity. Data are expressed as Mean $\pm$ SD.

Gastrointestinal Symptom	Pooled All Conditions	Baseline Exercise		Placebo Exercise		Nucleotide Exercise	
	Pre	Post	2 hrs post	Post	2 hrs post	Post	2 hrs post
Side stitch	0.0 $\pm$ 0.0	0.8 $\pm$ 1.6	0.0 $\pm$ 0.0	1.0 $\pm$ 1.7	0.0 $\pm$ 0.0	0.8 $\pm$ 1.7	0.0 $\pm$ 0.0
Nausea	0.0 $\pm$ 0.0	0.5 $\pm$ 0.8	0.2 $\pm$ 0.4	1.2 $\pm$ 2.0	0.1 $\pm$ 0.3	0.6 $\pm$ 1.0	0.1 $\pm$ 0.2
Bloating	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1 $\pm$ 0.5	0.0 $\pm$ 0.0	0.4 $\pm$ 1.0	0.0 $\pm$ 0.0
Urge to burp	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.3 $\pm$ 1.2	0.1 $\pm$ 0.2	0.0 $\pm$ 0.0	0.2 $\pm$ 0.5
Urge to Vomit	0.0 $\pm$ 0.0	0.5 $\pm$ 1.2	0.0 $\pm$ 0.0	0.4 $\pm$ 1.1	0.0 $\pm$ 0.0	0.6 $\pm$ 0.2	0.0 $\pm$ 0.0
Urge to Defecate	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.2 $\pm$ 0.4	0.2 $\pm$ 0.4
Diarrhoea	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Need to fluctuate	0.0 $\pm$ 0.0	0.3 $\pm$ 1.1	0.3 $\pm$ 1.0	0.2 $\pm$ 0.9	0.0 $\pm$ 0.0	0.2 $\pm$ 0.7	0.0 $\pm$ 0.0
Flatuation	0.0 $\pm$ 0.0	0.4 $\pm$ 1.2	0.3 $\pm$ 1.2	0.3 $\pm$ 1.2	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Stomach Cramps	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.1 $\pm$ 24	0.2 $\pm$ 0.7	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Stomach Upsets	0.0 $\pm$ 0.0	0.1 $\pm$ 0.2	0.0 $\pm$ 0.0	0.5 $\pm$ 1.2	0.1 $\pm$ 0.3	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0
Intestinal Cramps	0.0 $\pm$ 0.0	0.1 $\pm$ 0.2	0.0 $\pm$ 0.0	0.3 $\pm$ 0.7	0.0 $\pm$ 0.0	0.2 $\pm$ 0.5	0.0 $\pm$ 0.0
Dizziness	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Shivering	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Heart Burn	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

3.4.11 Serum Cortisol Data

Serum cortisol release increased significantly pre-to post RSA exercise returning toward baseline at 2 hours post activity ($P \leq 0.05$). No significant main effects or interactions between supplementation conditions were observed ($P \leq 0.05$).

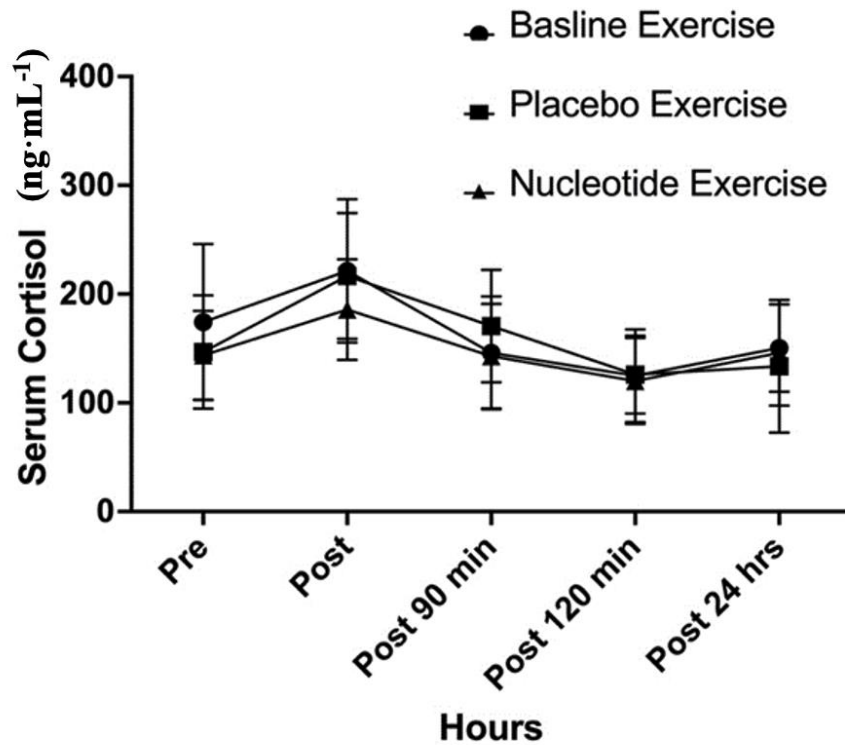


Figure 3.2.3 Serum cortisol ($\text{ng} \cdot \text{mL}^{-1}$) responses prior to and following the performance of the high intensity RSA protocol grouped by experimental treatment. Data are expressed as Mean $\pm$ SD. No Significant differences were determined.

3.5 Discussion

The aim of this study was to examine the effects of 30 days of purine and pyrimidine nucleotide supplementation compared to placebo (~2520 mg·day) on gastrointestinal permeability, damage, and symptom expression following an acute bout of high-intensity repeated sprinting under thermoneutral conditions. The principal findings indicate that, despite similar exercise intensity, performance, and psychophysiological responses across experimental conditions, there were no significant differences in gastrointestinal permeability, damage, or subjective gastrointestinal symptoms among recreationally active males. Overall, these results suggest that dietary nucleotide supplementation has no significant effect on various markers of gastrointestinal function following acute high-intensity sprint exercise.

Studies on dietary nucleotide supplementation in porcine, avian, bovine, aquaculture and human infant models indicate it has gastroprotective effects. These gastroprotective effects include improved gut morphology (e.g., increased villus height, crypt depth, villus wall thickness, villus count, and gastrointestinal surface area), enhanced intestinal blood flow, reduced oxidative stress, lowered systemic inflammatory response, elevated heat shock protein expression, maintained tight junction function, and stimulated enterocyte proliferation and repair (Uauy et al., 1994; Carver, 1999; Belo et al., 2006; Chen et al., 2011; Sauer et al., 2012; Al-Okbi, et al., 2020; Hossain et al., 2020; Upadhaya and Kim 2021; Valini et al., 2021). Conversely, in the absence or insufficient availability of dietary nucleotides, these gastroprotective pathways are compromised, resulting in both morphological and functional gastrointestinal tract impairment (Holden et al., 2004; Hess and Greenberg, 2012; Xu et al., 2017; Ding et al., 2021).

This study aimed to determine whether similar gastroprotective effects of nucleotide supplementation is observed in adult humans following an acute high-intensity exercise bout. The gastrointestinal barrier, comprising physical and chemical components, selectively permits nutrient and water absorption while preventing pathogenic and antigenic agents from crossing into systemic circulation (Lambert, 2008; Camilleri et al., 2012; Grootjans et al., 2016; Camilleri, 2019; Camilleri and Velda, 2021). To evaluate gastrointestinal barrier function, Dual Sugar Absorption Test (DSAT) was conducted to measure the translocation of L-lactulose and L-rhamnose marker probes. This test provides an index of small intestine barrier damage and gastrointestinal permeability by indicating disruptions in gastrointestinal mucosal integrity through paracellular and/or transcellular pathways (Lambert et al., 2008; Costa et al., 2017;

Vanuytsel et al., 2021). Increased appearance of these probes in systemic circulation suggests loss of gastrointestinal membrane integrity (Fleming et al., 1996; Grootjans et al., 2010; van Wijck et al., 2012; Vanuytsel et al., 2021). Further, I-FABP, an intracellular protein specific to the apical epithelial cells of the small and large intestine, was used as a marker of morphological injury to the intestinal villi. After injury to the small intestine, apical cells of the villus are damaged and shed releasing I-FABP into circulation, permitting the detection of circulating protein level (Pelsers et al., 2003; Pelsers et al., 2005; Thuijls, et al., 2011; van Wijck et al., 2011; Lau et al., 2016). Given I-FABP's approximately 11-minute half-life, its serum presence indicates recent, acute intestinal injury but not cumulative intestinal injury (van Wijck et al., 2012). The principal findings of this work indicates that no significant improvements to gastrointestinal permeability or damage, as shown by L/R ratio or I-FABP levels, across control, placebo, and chronic nucleotide-supplemented conditions following repeated sprint activity was detected. Both L/R ratio and I-FABP exhibited minor stepwise increases across trials [baseline control < placebo < Nucleotide-supplemented], indicating a slight, non-significant decline in GI barrier function and villus morphology post-RSA. This was observed despite similar RSA exercise intensity, workload, and physiological responses across conditions, including cortisol responses, which further corroborate load equivalence.

The lack of significant differences in L/R ratio, I-FABP, and gastrointestinal symptoms among control, placebo, and nucleotide supplemented groups suggests no detectable gastroprotective effect of nucleotide supplementation at rest (I-FABP, symptomology) or after exercise (L/R, I-FABP and symptomology). The observed, albeit non-significant, decline in gastrointestinal barrier integrity with nucleotide supplementation contradicts the hypothesis that chronic nucleotide ingestion would maintain or enhance gastrointestinal barrier function. Wide intra and inter-individual biomarker fluctuations are evident from the data particularly in I-FABP observed across trials. This to be expected particularly at rest, as the gastrointestinal barrier undergoes constant repair and replacement due to factors like digestion, blood flow, tissue hypoxia and immune activation (Camilleri et al., 2012; Colgan et al., 2016; Camilleri, 2019; Camilleri and Velda, 2021). L/R ratio follows a similar trend in literature, however, the present study's design does not permit an evaluation of resting L/R ratio stability or relative rest to exercise change in permeability, as it focused solely on post-exercise changes as such this is a limitation of the present experimental design. However, Hamzah et al. (2018) has reported wide intra-individual variances of L/R ratio and by implication barrier integrity at rest.

Present findings across all conditions should be contextualised within the broader literature, where present L/R ratios and I-FABP levels post-RSA exercise reflect a very low level of gastrointestinal disruption/damage compared to studies involving sustained endurance and intermittent exercise, which often report much higher L/R ratios and significant I-FABP elevations indicative of more extensive gut dysfunction. Typically L/R ratio and I-FABP increases have been reported after 60 minutes of cycling at 70% maximal power output (van Wijck et al., 2011; Sheane et al., 2018), treadmill running at 60% of $\dot{V}O_2$ max for 120 minutes (Axelrod et al., 2019), and treadmill running at 70%–75% $\dot{V}O_2$ max (Marchbank et al., 2011a; Zuhl et al., 2014a; Takata et al., 2022) or even 80% $\dot{V}O_2$ max (Pals et al., 1997; Davison et al., 2016; Kahru et al., 2017; Snipe et al., 2018; Ogden et al., 2021). Typically, post exercise L/R ratios during these protocols range from 0.07 to 0.97; a broad range. These findings are consistent with those observed during intermittent exercise. Pugh et al. (2017) and Jafari et al. (2022) reported significant increases in L/R ratio and I-FABP levels in male and female athletes performing 18 × 400 m running intervals at ~110%–120% of $\dot{V}O_2$ max. Similarly, Hamzah et al. (2018), Wallett et al. (2022), McKenna et al. (2022), and Chantler et al. (2022) examined these the same biomarkers in recreational athletes and rugby players across short (RSA: 4 × 5 × 6 s sprints with ~15–30 s recovery), medium (45 min), and long (~2 hours) intermittent exercise protocols. Collectively, these studies demonstrated that L/R ratios and I-FABP levels significantly increase with exercise duration and intensity. Notably, Pugh et al. (2017), Chantler et al. (2022), Jafari et al. (2022), and McKenna et al. (2022) observed marked elevations in gastrointestinal permeability (higher L/R ratio) and GI damage (elevated I-FABP) following exercise, with greater changes in the longer protocols compared to shorter RSA protocols, which showed no significant post exercise increases in L/R ratio or I-FABP (Hamzah et al., 2018; Wallett et al., 2022). In the present study, I-FABP levels followed a similar time course of increase and recovery as observed in other intermittent exercise models but exhibited a notably lower response magnitude compared to post-exercise values exceeding 1000-3000 pg.mL⁻¹ reported in previous studies (Pugh et al., 2017; McKenna et al., Snipe et al., 2018; Gaskell et al., 2020; Chantler et al., 2022).

Together, there is no indication gastrointestinal barrier integrity has been significantly breached across intervention conditions. The limited gastrointestinal disruption observed suggests that nucleotide supplementation minimally affects gastrointestinal barrier integrity or damage relative to placebo and control. Indeed, the post RSA exercise L/R ratio data and I-FABP data observed herein more closely reflect ‘resting’ values data seen in other studies. Lambert et al.

(2008) reported resting L/R ratios of 0.035 (range: 0.011–0.107), while Pals et al. (1997) noted values of 0.048 ± 0.009 . Similarly, Pugh et al. (2017) observed values of 0.031 ± 0.021 , and Zuhl et al. (2014; 2015) reported 0.0218 ± 0.008 and 0.02 ± 0.01 , respectively. Hamzah (2018), studying recreationally active participants, documented a resting L/R ratio of 0.05 ± 0.003 following a similar RSA protocol. These findings on GI permeability and damage biomarkers highlight potential limitations in using these parameters for cross-study comparisons. While L/R ratios appear numerically similar across studies, they may represent distinct physiological states of the gastrointestinal barrier. For example, post-exercise L/R ratio and I-FABP values in one study may align with resting values in another. Consequently, caution is warranted when interpreting absolute L/R ratio and I-FABP values for physiological significance across studies.

Whilst the objective markers of permeability and damage showed little change across the treatment conditions, it is the occurrence of gastrointestinal symptoms which primarily impact individuals during and following exercise. Subjective symptoms of gastrointestinal dysfunction such as abdominal cramps, diarrhoea, nausea, vomiting, and bloating are frequently reported, amongst endurance and intermittent trained athletes particularly where running is predominant modality (de Oliveira et al., 2014; Wilson, 2017; Costa et al., 2020; Pugh et al., 2023). Subjective gastrointestinal symptoms were assessed in this chapter using a multi-symptom VAS scale representing a range of upper and lower gastrointestinal symptoms which indicated minimal to low symptom severity across all conditions, despite the high-intensity nature of RSA activity (Lambert et al., 2008; Pugh et al., 2017; Wilson et al., 2018). Past studies have associated RSA protocol induced metabolic acidosis, hyperlactacidaemia and falls in pCO_2 with acute GI symptoms such as nausea in the immediate post exercise period (Merrells et al., 2019, Merrells et al., 2021). Despite the significant elevation in lactate post exercise and likely metabolic acidosis subjective symptom expression was not prominent in the present findings. This may reflect the retrospective recall of symptoms and VAS completion ~ 5-10 minutes post exercise and a reduction in perception with this delay. Merrell's et al. (2021) has also indicated perception of nausea declines in severity in the 6-minute period following RSA completion. Further, Merrell's et al. (2021) have noted the inclusion of active recovery in a protocol reduces gastrointestinal/nausea symptom expression relative to passive recovery. These factors may contribute to the low symptom scores reported after RSA performance.

The low levels of subjective symptoms reported following RSA exercise were not aligned with the objective changes in gastrointestinal permeability and damage biomarkers observed by

others. Pugh et al. (2017) describe a similar dissociation between symptoms and objective markers of gastrointestinal damage after a longer high intensity interval exercise protocol (18 x 400 m). Hamzah et al. (2018) also reported a lack of association following the same RSA protocol (6 × 35 m × 4 sets) used in this study. These findings align with previous reports of minimal or absent gastrointestinal symptoms across various exercise intensities, modalities, and environmental conditions (Lambert et al., 2008; Marchbank, et al., 2011a; van Wijck, et al., 2011a; Karhu, 2017; Gaskell et al., 2021; King et al., 2022). This dissociation may reflect the tendency of experimental studies to focus on small intestinal barrier function and damage (Smetanka et al., 1999; Lambert et al., 2008; Gill et al., 2015b; McKenna et al., 2017; Pugh et al., 2017; Costa et al., 2020), whereas symptom mapping and epidemiological data suggest gastrointestinal symptoms are predominantly associated with large intestine dysfunction (Lambert, 2004; Lambert et al., 2008; Gaskell et al., 2021; Wilson et al., 2023). These findings suggest that the relevance of correlating gastrointestinal symptoms with biomarkers warrants re-evaluation, potentially requiring more comprehensive markers of both small and large intestine damage to establish stronger associations.

The lack of nucleotide efficacy in the present study contrasts with the findings of the only other gastrointestinal human nucleotide trial. Dancey et al. (2006) reported an improvement in IBS related symptoms following nucleotide supplementation at a lower dose (1500 mg) but over a longer duration (56-day) using a purine and pyrimidine nucleotide composition like that of the present study. This discrepancy may be attributed to differences in participant characteristics, as Dancey et al. (2006) studied female participants clinically diagnosed with irritable bowel syndrome, whereas the current study involved young, healthy, predominantly male volunteers who were specifically screened to exclude such conditions. These findings may support the hypothesis that nucleotides become conditionally essential only under conditions of chronic or severe gastrointestinal stress.

The absence of significant objective and subjective gastrointestinal disruption across trial conditions in the present study and may be attributed to various RSA protocol-related factors that mechanistically influence the gut's response to exercise, as well as methodological factors related to the measurement of small intestinal barrier function and damage (Gaskell et al., 2020; Ribeiro et al., 2021).

Mechanistically, reductions in splanchnic blood flow and increases in gut thermal load (temperature), both of which are mediated by exercise duration and intensity, have been identified as primary contributors to exercise-induced gastrointestinal permeability and damage during prolonged, sustained exercise (Lambert, 2008; Costa et al., 2017; Pugh et al., 2017; Snipe et al., 2018c; Gaskell et al., 2020; Chantler et al., 2021). These factors are thought to drive increased intestinal permeability and damage by disrupting enterocyte tight junction proteins, inducing oxidative stress, releasing pro-inflammatory cytokines, impairing mucin secretion, causing enterocyte hypoxia, and activating immune responses (Glover and Colgan et al., 2011; Zuhul et al., 2014; Dokladny et al., 2016; Costa et al., 2017; Chantler et al., 2021).

In the present study, it remains unclear whether the lack of significant changes in gastrointestinal permeability, damage, and symptoms following the RSA trials reflects insufficient activation of these mechanisms due to the relatively low intensity and/or short duration of the RSA protocol and/or individual gastrointestinal adaptations. Endurance exercise activity is acknowledged to reduce splanchnic blood flow up to ~80% (Otte, et al., 2001; Perko et al., 2004; Crandall and Gonzalez-Alonso, 2010; van Wijck, et al., 2011). This reduction is inversely proportional to the percentage of maximal oxygen consumption ($\dot{V}O_2$ max) achieved during exercise performance and its duration (van Wijck et al., 2011). Splanchnic blood flow responses following RSA ‘intermittent exercise’ are less well described. The gastric tonometry assessments by which splanchnic blood flow is estimated requires a steady state to be established one which intermittent exercise does not facilitate (Otte et al., 1999; Cerný and Cvachovec, 2000; van Wijck, et al., 2011). As such, it is uncertain the amount to which splanchnic blood flow is impaired, the time over which the reduction occurs and remains in place during the present RSA protocol where the exercise is short and high intensity in nature. The oscillation between high and low intensity exercise during RSA activity may induce a similar pattern in splanchnic blood flow with hypoperfusion during the exercise activity followed re-perfusion within the rest period i.e., between reps or sets allowing blood flow redistribution back to the gut when exercise intensity is reduced.

Additionally, with a period of hypo-perfusion followed by a period of reperfusion in the enterocytes a perfusion-reperfusion hypoxic/oxidative stress response may be induced (Zheng, et al., 2015). Costa et al. (2017) has summarised the potential mechanistic pathway by which intestinal injury occurs from blood flow changes; an initial ischemia and a subsequent reperfusion stress upregulates NF- κ B gene expression within the epithelial cells, which signals

the release of pro-inflammatory cytokines such as interleukin (IL)-1 β , tumour necrosis factor (TNF)- α and interferon (IFN)- γ , and a subsequent local inflammatory cascade. Subsequently, the removal off hypo-perfusion ischemic stress and the restoration of inflammatory response, can lead to further intestinal damage and impairments in the integrity of the gastrointestinal barrier via disruption to tight junction protein integrity allowing barrier permeability to increase (Derikx et al., 2008b; Grootjans et al., 2010). This oxidative stress's extent depends on the duration and completeness of the blood flow restriction and individual prior exposure and compensatory adaptations. Grootjans et al. (2016) indicate that complete small intestinal ischemia for ~45 minutes results in higher transient levels of oxidative stress and gastrointestinal barrier failure, but not 30 minutes (Matthijsen et al., 2009) both of which may recover barrier function 120 mins after reperfusion (Schellekens et al., 2017). It is not clear if short periods of hypoperfusion (< 2 mins) as potentially experienced in the present RSA protocol can induce changes in the barrier permeability and damage; present data suggests not. Consequently, whether these RSA protocol effects acting together could impact gut barrier integrity in a manner which reflects that noted with longer exercise duration exercise models is unknown and should be quantified both in vitro and in vivo.

In addition to impairments in splanchnic perfusion, elevations in gut temperature are present and occur as function of individual responses to exercise intensity and duration (Racinais and Sawka, 2015). Elevations in small and large intestinal temperatures facilitated by exercise also compromise the integrity of the gastrointestinal barrier via similar mechanism to that observed with tissue ischemia (Lambert et al., 2002; Dokladny, et al., 2016; Costa et al., 2017; Snipe et al., 2018b). Pires et al. (2016) has indicated that exercise induced gastrointestinal permeability is subject to a 'temperature threshold' (38.0 °C-39.0 °C) which if exceeded for 'sufficient time' will increase gastrointestinal barrier permeability and villus apical cell damage. Core gastrointestinal temperature was not directly or continuously assessed in this study due the logistics of measuring it during the overground RSA protocol as such the relative contribution of hyperthermia either above or below these thermal threshold levels to the observed gastrointestinal permeability was assessed with a surrogate measure. Aural [tympanic] temperature was used as a marker of core temperature, average values whilst not different between conditions were lower than would be expected relative to normal values reported in literature. In this study, participants did not exceed the ~38.6°C threshold; the thermoregulatory strain of the RSA was determined to be low relative to the Pires et al. (2016), thermal damage model. It has been noted that tympanic temperature consistently under reports core temperature

estimates relative to rectal measures in exercising individuals (Casa et al., 2007; Huggins et al., 2012; Towey, et al., 2017). Aural temperature data in this chapter were reported, as measured, without correction, whilst some recommend a correction factor of $\sim 0.5\text{--}0.8\text{ }^{\circ}\text{C}$, inconsistencies in how much to correct for may result in errors measuring exercise-induced hyperthermia, especially at higher T-core levels (Huggins et al., 2012; Slaughter et al., 2018; Morrissey et al., 2021). Further, Kenny et al. (2009), reports that the intermittent nature of the RSA task facilitates a greater increase in evaporative heat loss following the initial exercise bout(s) such that the amount of heat stored in the successive exercise bouts is reduced by as much as 40–60% under compensable conditions as performed in this study. This would theoretically allow for gastrointestinal temperature to decline upon exercise cessation before rising again upon its recommencement. It is likely that the hyperthermic effect of the RSA protocol did not exceed the modelling threshold for gastrointestinal permeability and damage induction as set out by Pires et al. (2016).

Combined these blood flow and temperature considerations may be indicative of an RSA protocol threshold effect in terms of its ability to induce damage to the gut. The present protocol required short bursts of $\sim 5\text{--}6$ seconds supra maximal efforts followed by ~ 15 second of easy sub-maximal-running to return to the start line for the subsequent sprint effort. This equated to about 30 seconds of supra maximal and 90 seconds of sub-maximal effort in each set (1:3 work: rest ratio) followed by 5 minutes of rest delivered between each of 4 sets. In total, physical work time was about ~ 8 minutes over the course of the protocol, these factors of a short but intense exercise bout, active sub-maximal recovery and extended intra-session passive rest periods may be sufficient to explain the low gastrointestinal permeability, damage and symptomology responses induced by the RSA protocol relative to that observed during sustained high intensity exercise in the literature. These non-significant changes in gastrointestinal permeability following the short RSA activity are consistent with some reports that indicate high intensity exercise does not always cause gastrointestinal dysfunction (van Wijck et al., 2014; Yeh et al., 2013; Hamzah et al., 2018; Wallett et al., 2022). Therefore, the ‘total physiological load applied’ in the individual participants by the exercise protocol would seem critical in determining whether the homeostatic limits/threshold (combined blood flow/thermoregulatory challenge/hypoxia etc.) of the gut are maintained or exceeded. The occurrence of gastrointestinal dysfunction through barrier integrity breakdown would reflect the amalgamated effects of these processes. The present RSA exercise model must be considered for its ability to exceed these limits and facilitate disruption to gastrointestinal

barrier. Present data indicates the protocol was likely of insufficient intensity and duration to damage/alter gastrointestinal barrier integrity to the point that significantly increased the translocation of L/R across the gastrointestinal barrier, elevated I-FABP and symptom expression significantly in the participants recruited to the study. Hamzah et al. (2018) has reported following an identical RSA protocol and permeability assessment approach no significant increases in post exercise gastrointestinal permeability, I-FABP release and symptom expression relative to resting values. Further support for the present RSA model potentially not being disruptive may come from the observations of Wallett et al. (2022) who noted that only when short RSA activity (like the present work) is undertaken in uncompensable heat stress (40°C) is the combination of heat stress and exercise stress synergistic and sufficient to significantly increase biomarkers of gastrointestinal permeability and damage.

While protocol-related issues are considered potential factors contributing to the observed outcomes, it should also be noted that the participants in this study were diverse in terms of their fitness levels, training status, and activity patterns. This diversity suggests that some degree of training-related adaptation to repeated sprint activity (RSA) as well as gastrointestinal training-related adaptation may have occurred (Rehrer et al., 1990; Costa et al., 2017; Miall et al., 2017; Rehrer et al., 2021). The gastrointestinal tract exhibits substantial plasticity, adapting to a range of stressors that can alter both its morphology and function, as demonstrated in animal models (Mosser et al., 2017; Hossain et al., 2020; King et al., 2022; Ding et al., 2021).

The training status of the individual/athlete may therefore play a critical role in predisposing them to the expression of objective and subjective markers of gastrointestinal dysfunction (Costa et al., 2017; Guy, et al., 2016; Pugh et al., 2023). For example, Lu et al. (2014), using a mouse swim model, demonstrated that one week of swim training for 30 minutes per day attenuated increases in gastrointestinal permeability. Similarly, Miall et al. (2017), in a human model, reported a reduction in gastrointestinal symptomology following repeated exposure to exercise training. These findings align with those of Jafari et al. (2022), who showed that eight weeks of training in female athletes attenuated post-exercise increases in gastrointestinal biomarkers after an acute bout of high-intensity interval training (HIIT) [18 x 400m at 120% $\dot{V}O_2$ max]. Additionally, Wallett et al. (2021) observed that even a short period of HIIT training (5 days) in the heat reduced gastrointestinal damage following RSA exercise under similar

conditions. Moreover, Kierns et al. (2020) and Wallett et al. (2022) reported improved tolerance to gastrointestinal disturbances and a reduction in the appearance of gastrointestinal biomarkers in well-trained runners, which reflected training-induced adaptations compared to less well-trained individuals. However, the findings are mixed, other studies have reported minimal or no training effects on reducing gastrointestinal permeability and damage following endurance or intermittent exercise training in humans (Sumi et al., 2023; Chantler et al., 2021; King et al., 2022).

The implications for the present chapter are that the heterogeneous exercise and training status of the participants may have contributed to the observed variations in gastrointestinal permeability and I-FABP concentrations. These variations may not solely be a function of the nucleotide intervention but rather the result of individual adaptive responses to exercise training. Although a repeated-measures design was implemented, the training status varied significantly across individual participants, likely contributing to the high variability observed in the biomarkers. Furthermore, habitual physical activity was not restricted throughout the experimental phases, which spanned several months. As such, individual differences in exercise volume and intensity among participants may have resulted in gastrointestinal adaptations independent of the supplementation protocol. The present study cannot rule out the possibility that gastrointestinal biomarker and symptomology data were influenced by the progression of time and training-related adaptive responses in the individual participants' gastrointestinal systems. Additionally, the progression of time implies changes in the gastrointestinal epithelial tissue, mucin depth, and tight junction protein expression, all of which turn over approximately every 72 hours in humans. In animal models, enterocyte turnover rates vary, with mice showing a turnover of around 2.81 days, rats around 2.37 days, and humans exhibiting a longer turnover of 3.48 days. However, it should be noted that each individual participant may express a slightly different time sequence that may be longer or shorter as noted in animal models (Darwich et al., 2014). The extent of these changes and point in the turnover cycle may have varied at the time of each measurement. Although the counterbalanced experimental design aimed to account for such effects, the extent to which these factors impacted the data outcomes remains unknown.

Furthermore, the metrics used to assess gastrointestinal barrier integrity, such as the lactulose-to-rhamnose (L/R) ratio and intestinal fatty acid-binding protein (I-FABP), as well as the differences observed across studies, may reflect methodological inconsistencies in how

gastrointestinal permeability and damage are measured (Pugh et al., 2017; Chantler et al., 2021; Vanuytsel et al., 2021). These markers were originally developed for clinical applications to represent gastrointestinal dysfunction (Thuijls et al., 2011; Vojdani, 2013; Costa et al., 2017; Costa et al., 2020). However, their application in research has led to variability in methodological approaches across the exercise science literature. Such differences include the type of dual-sugar probes utilised, the timing of probe administration (e.g., before, during, or after exercise), the resolution and detection techniques employed to measure the sugar probes in biological fluids (e.g., HPLC reverse phase versus HPLC-MS), the type and sensitivity of I-FABP antibodies, and variations in sample collection protocols and media (e.g., urine versus serum versus plasma). These inconsistencies complicate the comparison and interpretation of quantitative permeability indices, such as L/R ratios and I-FABP (van Wijck et al., 2012; Vojdani, 2013; Bischoff et al., 2014; Chantler et al., 2021; Wallett et al., 2021; Martinez et al., 2022; Pugh et al., 2023). Additionally, the use of these markers in exercise research is derived from their clinical utility. However, their reliability, sensitivity, specificity, and normal ranges have not been adequately established in healthy, athletic populations. This lack of standardisation presents a significant limitation when considering the primary outcome variables of this study, which aim to reflect the efficacy of nucleotide supplementation and its effects on gastrointestinal barrier integrity.

The current findings, which indicate no significant effect of nucleotide supplementation on gastrointestinal permeability and I-FABP responses, contrast with the effects observed in studies employing other nutritional countermeasures. These studies have reported reductions in gastrointestinal barrier damage following exercise through various interventions. Acute supplementation with glutamine (Pugh et al., 2017), bovine colostrum (Marchbank et al., 2011), zinc carnosine (Davison et al., 2016), L-citrulline (van Wijck et al., 2014), and carbohydrates (Lambert et al., 2001; Costa et al., 2020) has demonstrated reduced gastrointestinal permeability and/or damage during single bouts of endurance exercise in thermoneutral and hot conditions (>30°C). However, there is currently no available data on the effects of these interventions during short-duration repeated sprint activity (RSA), as investigated in the present study.

Among these nutritional strategies, glutamine and bovine colostrum/zinc carnosine supplementation are predominant. Glutamine, a conditionally essential amino acid required for purine and pyrimidine nucleotide synthesis, has been shown to reduce intestinal injury at rest

(Mondello et al., 2010; Soares et al., 2014) and decrease intestinal permeability during exercise in thermoneutral and hot environments (Zuhl et al., 2014; Zuhl et al., 2015). Pugh et al. (2017) demonstrated that acute glutamine supplementation significantly reduced post-exercise gastrointestinal permeability and I-FABP concentrations in a dose-dependent manner following 18×400 m runs at 120% $\dot{V}O_2$ max. Similarly, Takata et al. (2022) reported that combined supplementation of cystine and glutamine attenuated the elevation of I-FABP compared to placebo after a 60-minute run at 75% $\dot{V}O_2$ max. Additionally, 14 days of supplementation with bovine colostrum and/or zinc carnosine ($20 \text{ g} \cdot \text{day}^{-1}$) has been shown to attenuate increases in gastrointestinal biomarkers, such as L/R ratios, tight junction proteins, and I-FABP, following short-duration (<20 minutes) high-intensity running (80% $\dot{V}O_2$ max), 1-h run (70% $\dot{V}O_2$ max) in the heat (30 °C/60% RH). (Marchbank et al., 2010; Marchbank et al., 2011; Davison et al., 2016; March et al., 2019). In vitro and animal studies further demonstrate that glutamine alone or in combination with bovine colostrum and zinc carnosine protects intestinal epithelial cells from reperfusion injury, mitigates oxidative stress, promotes epithelial cell proliferation, and increases villus height, thereby preserving tight junction protein integrity (Marchbank et al., 2010; Mondello et al., 2010; Davison et al., 2016; Wu et al., 2018; Deters and Saleem, 2021). These effects are likely mediated by glutamine's role as a precursor for purine and pyrimidine synthesis and its ability to enhance enterocyte proliferation and repair (Kim and Kim 2017; Deters and Saleem, 2021). Bovine colostrum is also enriched with nucleotides, particularly cytidine monophosphate (CMP), uridine monophosphate (UMP), adenosine monophosphate (AMP), and guanosine monophosphate (GMP) which may contribute to the noted effects on the gut (Mineguchi et al., 2021). However, disentangling the specific contributions of these components from such a complex "biological mix" is challenging and requires sophisticated experimental designs, particularly in vivo. (Mineguchi, et al., 2021). Conversely, other studies have reported no significant improvements in gastrointestinal permeability or I-FABP release with chronic bovine colostrum or acute glutamine supplementation across a range of exercise durations and environmental conditions. These discrepancies likely reflect variations in exercise intensity, duration, environmental conditions, supplementation dose, participant heterogeneity, and the individual participants gastrointestinal morphological and functional status (Mc Kenna 2017; Oliver et al., 2019; Osbourne et al., 2019; Ogden et al., 2021; Kierns et al., 2021; Ogden et al., 2022).

Several additional mechanisms may explain the lack of detectable effects of nucleotide supplementation on gastrointestinal barrier function in the present study, beyond the protocol-

and population-related factors previously discussed. The dose and formulation of ingested nucleotides, as well as their absorption and utilisation, are critical in determining their biological effects on the gastrointestinal tract. Nucleotides are primarily absorbed in the proximal small intestine, where they are hydrolysed by alkaline phosphatase and nucleosidase to nucleosides before being absorbed into enterocytes via nucleoside transporters (Carver and Walker, 1995; Pastor-Anglada et al., 2018; Wright and Lee, 2020). Hess and Greenberg (2012) and Ding et al. (2021) reported that approximately 50% of dietary nucleotides are retained and utilised by enterocytes for proliferation, repair, and cell signalling rather than systemic distribution. This preferential retention in enterocytes occurs because the small intestine has limited capacity for de novo purine nucleotide synthesis due to low expression of the key enzyme glutamine phosphoribosyl aminotransferase (Ding et al., 2021). Furthermore, the pyrimidine salvage pathways in the small intestine are inefficient due to limited expression of hypoxanthine-guanine phosphoribosyltransferase, making them rate-limiting for enterocyte proliferation and repair (LeLeiko et al. 1979). The exogenous dose and ratio of purine-to-pyrimidine nucleotides administered and absorbed are therefore critical for maintaining small intestinal morphology and function, including the assembly, stability, and phosphorylation of tight junction proteins, which regulate barrier permeability (LeLeiko et al., 1979; Uauy et al., 1994; Hess and Greenberg 2012; Daneshmand et al., 2017; Ding et al., 2021; Gopi et al., 2021).

Animal studies have demonstrated that dietary nucleotides upregulate tight junction proteins such as claudin-1, claudin-3, and zonulin-1, and enhance intestinal barrier integrity (Che et al., 2016; Wang et al., 2016; Jang and Kim, 2019). For example, Jia et al. (2016) showed in vitro that nucleotides increase claudin-4 phosphorylation and its interaction with zonulin-1 in CaCo-2 cell lines, thereby maintaining barrier function. Additionally, studies in porcine (Yang et al., 2014) and rodent models (Peng et al., 2017) have reported that dietary nucleotides attenuate the downregulation of tight junction proteins [claudin 1/occluding 1] and enhance their phosphorylation under stress conditions, preserving gastrointestinal barrier integrity. Mechanistically, nucleotides may achieve these effects by upregulating enterocyte gene expression and wound-healing pathways, as well as enhancing tight junction protein interactions (Jia et al., 2016; Lee and Kim, 2018; Suzuki et al., 2020). In the present study, circulating tight junction protein expression was not assessed, partly because systemic blood-based markers lack specificity and cannot attribute changes to specific gastrointestinal regions

without tissue immunohistochemistry. This limitation highlights the challenges in accurately determining tight junction protein dynamics in human models.

The optimal nucleotide dose required to maintain and/or enhance gastrointestinal morphology and function in humans remains undetermined. In contrast, data from animal models related to gastrointestinal development and growth is well-established (Daneshmand et al., 2017; Goppi et al., 2021; Goppi et al., 2023). Nucleotides are considered "conditionally essential" nutrients because their availability tends to be insufficient under stress conditions (Carver, 1999; Sánchez-Pozo et al., 2002). Consequently, the exogenous nucleotide dose required likely varies depending on the rate of enterocyte proliferation, damage, and repair necessitated by the imposed level of individual stress, as observed across both human and animal models. Meng et al. (2017) reported that a nucleotide dose of $0.3 \text{ g}\cdot\text{kg}^{-1}$ enhanced enterocyte proliferation, villus height, thickness, and surface area in fish, whereas a higher dose of $1.5 \text{ g}\cdot\text{kg}^{-1}$ caused oxidative stress and morphological damage to the small intestine. Similarly, Hossain et al. (2016) observed growth impairments at the $1.5 \text{ g}\cdot\text{kg}^{-1}$ nucleotide dose in an aquaculture model. Conversely, Selim et al. (2019) found that dietary nucleotide supplementation at $1.5\text{--}2.5 \text{ g}\cdot\text{kg}^{-1}$ produced beneficial effects on growth across other fish species. In avian and porcine models, nucleotide doses ranging from $0.05\text{--}0.25 \text{ g}\cdot\text{kg}^{-1}$ demonstrated anti-inflammatory properties and enhancements in intestinal growth (Jang and Kim, 2018). Perricone et al. (2020) noted that a dose of $0.8 \text{ g}\cdot\text{kg}^{-1}$ was required in pigs to elicit similar effects, while Keruz et al. (2020) reported that $0.3 \text{ g}\cdot\text{kg}^{-1}$ was effective in broiler chickens, promoting anti-inflammatory responses and gut proliferation. In a dose-response study on chicks, Goppi et al. (2023) observed improvements in gut function across a range of doses ($0.5\text{--}1.5 \text{ g}\cdot\text{kg}^{-1}$), with the highest dose eliciting the greatest effect. In a bovine model, Maggiolino et al. (2023) reported that a daily dose of $2.5 \text{ g}\cdot\text{kg}^{-1}$ enhanced gastrointestinal development and function. Furthermore, Belo et al. (2006) demonstrated that oral delivery of mixed nucleotides equivalent to $4\text{--}16 \text{ mg}\cdot\text{mL}^{-1}$ of gastric fluid reduced NSAID-induced gut ulceration by 60% in a rodent gastric damage model. These findings suggest species-specific differences in nucleotide requirements for optimal gastrointestinal function. Thus, nucleotide dosing must be carefully optimised, as either insufficient or excessive nucleotide concentrations may compromise growth and function in a species-dependent and or individual manner (Peres and Oliva-Teles, 2003; Oliva-Teles et al., 2006; Barros et al., 2015).

In adult humans, the dose-response relationship required to improve gastrointestinal morphology and function has not yet been defined. Speculatively, using the dosing model of Belo et al. (2006) as a reference, participants in the present study ingested 840 mg of nucleotides per dosing time point (three times daily). Assuming a gastric volume of approximately 100–200 mL at the time of ingestion, this would result in a nucleotide concentration of 4.2–8.4 mg·mL, similar to the concentrations shown to confer gastroprotective effects in animal models. Dancey et al. (2006), in the only adult human trial investigating nucleotide supplementation and gastrointestinal function, administered 1500 mg per day (three doses of 500 mg) for 56 days. This dosing regimen provided gastric concentrations of approximately 2.5–5.0 mg·mL (assuming a gastric volume of ~100–200 mL) and was associated with reductions in the clinical expression of IBS symptoms. In the present study, participants consumed 2520 mg of nucleotides daily for 30 days. However, this dosing regimen did not result in significant effects on gastrointestinal permeability, damage, or symptom expression compared to control and placebo conditions. These findings suggest that the optimal (individualised) nucleotide dose and timing required to improve gastrointestinal morphology and function remain unclear and warrant further investigation. It is possible that the administered nucleotide dose was excessive, as higher doses have been associated with a stepwise increase in gastrointestinal damage under certain conditions. These would mirror the trend seen in L/R ratio data presented herein.

In addition to dose, the relative formulation of dietary nucleotides ingested may also play a critical role in determining their effects on the gastrointestinal system. Similar considerations have been highlighted in other tissue models, such as muscle myotubes, where growth outcomes varied based on the relative formulation of purine versus pyrimidine nucleotides provided (Hossain et al., 2020; Uberti et al., 2020; Nakagawara et al., 2022). In porcine models, Bustamante et al. (1994) and Gao et al. (2021) reported that supplementation with purine and pyrimidine nucleotides increased the available gut nucleotide reservoir, which supported improved intestinal morphology and function. Additionally, the synthesis of mucus, an essential component of the gut epithelium that forms a protective layer to block or limit luminal antigen access, was enhanced by a mixed nucleotide diet (Wu et al., 2018; Fernández-Tomé et al., 2021). Similarly, other studies have noted improved mucin gene expression, enterocyte maturation and repair, increased sodium transporter density, and elevated enterocyte enzymatic activity following supplementation with purine and pyrimidine nucleotides compared to a

nucleotide-free diet (Che et al., 2016; Reda et al., 2018; Wu et al., 2018; Patterson et al., 2019; Mohamed et al., 2020; Li et al., 2022).

Belo et al. (2006) investigated the effects of individual nucleotides versus mixed formulations on rat small intestinal (IEC6) and human colonic (HT29) cells. Their findings indicated that adenosine monophosphate (AMP), cytidine monophosphate (CMP), and inosine monophosphate (IMP) individually stimulated migration of HT29 cells, while guanosine monophosphate (GMP) did not. In IEC6 cells, CMP, GMP, and IMP had no effect on proliferation, whereas AMP and a nucleotide mixture impaired proliferation, suggesting a potential cell-specific response to the type of nucleotide administered. Furthermore, Belo et al. (2006) reported synergistic effects when purine and pyrimidine nucleotides were combined, enhancing cell migration and wound healing responses compared to individual nucleotides. Purine and pyrimidine nucleotides, particularly those containing adenosine and uridine, have also been shown to reduce gut inflammation by inhibiting NF- κ B gene expression in epithelial cells and reducing pro-inflammatory cytokine production (e.g., IL-1 β , IL-6, and TNF- α). These nucleotides also promote anti-inflammatory cytokine production (e.g., IL-10 and TGF- β) and suppress T cell and macrophage activity, which are central to the enterocyte inflammatory response (Schenk et al., 2008; Trabanelli et al., 2012; Hess and Greenberg, 2012; Di Virglio et al., 2017; Cheng et al., 2020; Ding et al., 2021). This is of interest as exercise, activity upregulates this pathway (Costa et al., 2017) and may suggest a mechanistic avenue for nucleotide efficacy following ‘damaging exercise’.

Collectively, these findings suggest that mixed nucleotide formulations may have superior effects on the gastrointestinal system compared to individual nucleotide formulations. However, this perspective is not universally supported. Several recent studies using animal and aquaculture models have reported that individual purine or pyrimidine nucleotide supplementation, rather than mixed formulations, may yield greater improvements in growth and stress tolerance (Song et al., 2012; Hossain, 2016; Hossain, 2020). Guo et al. (2016) and Mok et al. (2019) specifically evaluated intestinal villus development in shrimp and found that individual nucleotide supplementation was more effective than mixed formulations. These discrepancies may reflect species differences, age, individual stress levels, duration of exposure, and other external factors that complicate the determination of optimal nucleotide composition and dosing (Belo et al., 2006; Kim and Kim, 2017; Kamel et al., 2021; Valini et al., 2021).

In the present study, the nucleotide formulation ingested was pyrimidine-based rather than purine-based. This formulation did not result in detectable changes in individual gut permeability, damage responses, or symptom expression following RSA activity. Consequently, it is not possible to confirm whether this formulation is optimal for maintaining gastrointestinal barrier function in humans. Enterocytes have a high proliferative rate, requiring sufficient nucleotide availability for nucleic acid synthesis, [which depends on absorption, de novo synthesis, and salvage of nucleotides] all highly energy-dependent processes (Saviano et al., 1981; Walker, 2006). The de novo purine biosynthetic pathway requires approximately 50% more ATP (6–7 ATP molecules) compared to pyrimidine biosynthesis (3–4 ATP molecules) and is a more complex metabolic process (Carver and Walker, 1995; Sánchez-Pozo et al., 2002). Thus, the balance of purine and pyrimidine nucleotides provided may influence these processes. A purine-focused nucleotide formulation could potentially reduce the metabolic energy cost of de novo synthesis, thereby enhancing energy availability for enterocyte proliferation and growth (Mok et al., 2021). However, Daneshmand et al. (2017) observed that a pyrimidine nucleotide supplement improved small intestinal development and function in a broiler chicken model, likely due to the higher pyrimidine bioavailability. In the current RSA model, pyrimidine supplementation may have been surplus to endogenous functional demand for enterocyte proliferation and repair, given the limited stress or individual responses to that imposed stress.

Both nucleotide dose and formulation appear critical to their effectiveness, but the mode of administration also plays a significant role in gastrointestinal outcomes. Belo et al. (2006) reported that oral nucleotide delivery reduced gastric damage by up to 60% in a rodent model, whereas subcutaneous delivery increased gastric damage. This suggests that the gastroprotective effects of nucleotides are mediated through local rather than systemic mechanisms in rats. In humans, nucleotide delivery is typically achieved orally or sublingually (McNaughton et al., 1997; Ostijic et al., 2012). In the present study, oral delivery likely facilitated the local availability of nucleotides in the proximal small intestine, with absorption mediated by nucleoside transporters expressed in the apical, epithelial, and basolateral membranes of enterocytes (Pastor-Anglada et al., 2018). These observations highlight the importance of delivery routes in determining the efficacy of nucleotides across different models, cell types, and organ systems.

3.6 Conclusions

In conclusion, this study represents the first investigation into the effects of chronic dietary nucleotide supplementation on gastrointestinal function following repeated sprint activity (RSA) exercise. The findings indicate that nucleotide supplementation did not confer gastroprotective effects, as there were no significant differences in gastrointestinal permeability, damage, or symptomology among the control, placebo, and nucleotide conditions post-exercise. These results suggest that the morphological and functional adaptations frequently reported in animal models [such as enhanced barrier integrity and resistance to mucosal damage] were not evident under the dosing and formulation regimen used in this study. Alternatively, given the robust evidence from animal studies demonstrating functional adaptations within similar time--frames, these results may reflect insufficient physiological stress imposed upon the gut to elicit such adaptive responses. The RSA exercise model employed in this study may not have induced a sufficiently high level of physiological or environmental challenge to disrupt the homeostatic balance of the gut and reveal potential gastroprotective effects of nucleotide supplementation. As such, it can be concluded that under conditions of "suboptimal stress load", [characterised by RSA in a thermoneutral environment and performed by an exercise-habituated population] the individuals gut maintains its inherent homeostatic stability and self-repair capacity, thereby limiting the need for exogenous nucleotide provision.

Previous research has demonstrated a link between exercise and increased gastrointestinal dysfunction, but these effects have typically been observed under more strenuous exercise conditions. Specifically, these studies often employed continuous or intermittent exercise protocols at intensities exceeding 80% $\dot{V}O_2$ max, with longer exercise durations and/or exposure to environmentally challenging conditions such as heat stress (Costa, 2017; Chantler et al., 2021; Pugh et al., 2023). The inability of the present RSA exercise protocol to exceed the participant's homeostatic capacity of the gut must therefore be considered a key limitation of this study. Future investigations into the role of dietary nucleotides in supporting GI function should re-evaluate the total physiological and environmental loading applied during exercise. An appropriate challenge that sufficiently disrupts gut homeostasis is critical for testing the potential adaptive and protective effects of nucleotides. Only under such conditions will it be possible to optimise the dosage and formulation of nucleotide supplementation and determine whether a "conditionality threshold" for nucleotides exists in humans, as suggested by findings in animal models.

Chapter 4

Effect of 30-Days Dietary Nucleotide Supplementation upon the Induction and Elicitation of Cutaneous Hypersensitivity in Response to Acute Repeated Sprint Exercise. (Study 3)

Empirical studies in both animal and human models have established the immunomodulatory effects of nucleotide supplementation. These investigations demonstrate that dietary nucleotides can enhance both innate and adaptive immune responses following immunological challenges. Such findings highlight the potential of nucleotides as functional agents in promoting immune resilience, particularly under physiologically demanding conditions. High-intensity exercise is known to transiently suppress immune function, increasing susceptibility to infection and delaying recovery. Nucleotide supplementation may help maintain immunological vigilance during such periods, supporting consistent training and reducing illness-related interruptions to training. As key components in cellular signalling and nucleic acid synthesis, nucleotides may enhance immune cell proliferation and responsiveness during both acute and chronic stress. To explore this further, the following chapter will examine the immunomodulatory effects of nucleotides following high intensity repeated sprint exercise using the Contact Hypersensitivity (CHS) in vivo model. This approach assesses localised T-cell mediated immune responses to a chemical allergen, allowing for the evaluation of immune activation and memory. Such findings may further shift the conceptual framework of nucleotide use from solely performance enhancement (Chapter 2) to a broader adaptogenic paradigm of immunological resilience.

4.1 Abstract

This chapter investigated the effects of chronic oral ingestion of purine and pyrimidine nucleotides on the induction and elicitation of T-cell-mediated immunity to a novel chemical antigen, diphenylcyclopropenone (DPCP). The study utilised the contact hypersensitivity (CHS) model, a clinically relevant in vivo measure, following the performance of high-intensity repeated sprint exercise. Twenty-four male recreationally trained individuals [age, 22.7 ± 3.7 years; height, 177.7 ± 5.6 cm; weight, 74.4 ± 7.6 kg; 49.0 ± 5.1 mL·kg⁻¹·min⁻¹] were allocated into either a placebo (P_{exe}) or nucleotide (N_{exe}) supplemented group using a minimisation of difference matched pairs design. Each participant undertook high intensity intermittent repeated sprint exercise (RSA) [4 set x [6x35 m @ 15 sec active recovery] with a 5 min passive recovery between sets following 30 days of oral purine and pyrimidine nucleotide (2520mg) or placebo ingestion. Ingestion of nucleotide supplement was stopped on the night prior to CHS protocol commencement. Each participant received a primary DPCP exposure (sensitisation dose), 20 min after exercise by attaching a patch containing 22.8 µL of 0.125% DPCP in acetone to the lower back for 48 hours. Four weeks later, participants in both groups received a low, dose-response series of DPCP challenge (elicitation) on their upper arm, which was determined 48 h after exposure as a clinical index outcome measure of cell mediated immunity and inflammation as tissue oedema (skinfold thickness), redness (erythema), melanin change, total cutaneous hypersensitivity response area. Heart Rate, lactate, RPE, and sprint performance times were collected as an index of exercise performance. Serum cortisol was utilised as a marker of stress responses via ELISA and uric acid as a marker of purine related metabolism. Data indicate exercise performance equivalence across all conditions: Sprint Times (N_{Exe} 6.5 ± 1.1 s vs P_{Exe} 6.2 ± 0.5 ; $P \geq 0.05$), Fatigue Index (N_{Exe} $21.2 \pm 14.1\%$ vs P_{Exe} $17.6 \pm 6.1\%$; $P \geq 0.05$), Lactate (N_{Exe} 11.2 ± 2.2 mmol·L⁻¹ vs P_{Exe} 10.6 ± 2.2 mmol·L⁻¹ $P \geq 0.05$). CHS induced dose response clinical markers were not significantly different for Erythema (redness) (N_{Exe} 13.2 ± 3.6 vs P_{Exe} 14.7 ± 3.6 AU; $P \geq 0.05$), Melanin (N_{Exe} 36.9 ± 3.8 vs P_{Exe} 36.1 ± 4.4 AU ; $P \geq 0.05$), tissue oedema change (N_{Exe} 0.65 ± 1.48 mm vs P_{Exe} 0.27 ± 2.97 ; $P \geq 0.05$), and total cutaneous hypersensitivity response area (N_{Exe} 25505 ± 37504 AU vs P_{Exe} 29014 ± 31024 AU). Similarly, no differences in Cortisol (24 hours) and Uric acid responses were observed. (N_{Exe} 152.9 ± 39.2 vs P_{Exe} 135.1 ± 46.4 ; $P \geq 0.05$) or (N_{Exe} 294.4 ± 70.8 vs P_{Exe} 271.8 ± 79.4 ; $P \geq 0.05$). Data suggest that, compared to placebo supplementation, a 30-day 2520 mg regimen of nucleotide supplementation does not result in differences in the induction and elicitation of CHS immunity following exposure to and recall of DPCP, after performing high-intensity repeated sprint exercise.

4.2 Introduction

The human immune system serves as the primary defence mechanism against pathogens, providing for host immune surveillance, playing a pivotal role in maintaining protection against external and internal challenge (Yatim et al., 2015; Gleeson et al., 2020). Exercise modulates human immune function, revealing a complex response characterised by alterations in immune cell population: numbers, function, signalling pathways, and systemic trafficking responses (Nieman, and Pedersen, 1999; Walsh et al., 2011; Simpson et al., 2020). The impact of exercise on immune function is often conceptualised within the "open window hypothesis" (Pedersen and Ullum, 1994; Nieman and Pedersen, 1999; Peake et al., 2017; Kakanis et al., 2020). This hypothesis suggests that intense or prolonged bouts of exercise may transiently alter immune responses, including changes in the numbers and function of both non-specific innate and specific adaptive immune cells and their sub-lineages. These alterations may involve shifts in cytokine and chemokine signalling, increased pro-inflammatory activity, and downregulation of anti-inflammatory pathways, potentially rendering individuals more susceptible to infections.

Conversely, moderate-intensity exercise is associated with beneficial immunomodulatory effects (Peter, 1997; Schwellnus et al., 2016; Soligard, et al., 2017; Simpson et al., 2020). The relationship between exercise, immune function, and disease expression is highly context-dependent, influenced by factors such as exercise modality, intensity, duration, and frequency. These factors drive physiological and endocrine-induced changes in blood flow and tissue perfusion, leading to differential redistribution and activation of immune cells. Additional modifiers include age, environmental conditions, nutrition, psychological stress, sleep, and individual characteristics. (Gleeson 1997; Nieman and Pederson, 1999; Simpson et al., 2015; Peake et al., 2017; Simpson et al., 2020). Acute supra-maximal intensity interval exercise either as single or repeated bout(s) may disrupt both innate, secretory, and cellular immune function in the hours and days post exercise cessation (Fahlman et al. 2001; Hall et al. 2007; Mathes et al., 2017; Chastin et al., 2021; Souza et al., 2021). In the context of the upsurge in HIIT training and the demands for repeated sprint activity during intermittent team sports such observation may impact training efficacy and resistance to infection.

Nutrition plays a crucial role in modulating immune function and mitigating stress induced immunosuppression by facilitating increased immune resilience through influencing cell trafficking, activation and proliferation, cytokine production (Venkatraman and Pendergast, 2002; Childs et al., 2019). Purine and pyrimidine dietary nucleotides in particular have been

shown in animal and human models to be critical to these functions as well as acting to maintain/improve disease resistance, and reduce antibiotic requirements to experimentally induced and naturally occurring disease when both subject to physiological, cellular, environmental stress (Van Buren et al., 1994; Maldonado, 2001; Gil 2002; Carver, 2003; Yau et al., 2003; Li et al., 2007; Verkerk and Gil, 2009; Hess and Greenberg, 2012; Jang and Kim, 2019 Khammel et al., 2021). The cells of the immune system express a high proliferative rate and a limited ability to synthesize nucleotides, therefore establishing in these cells the ‘conditional essentiality’ principle i.e., they require additional exogenous nucleotide provision to meet demand that cannot be met endogenously (Carver and Walker, 1995; Gimble et al., 2001) particularly when both animal and humans are subject to increased stress (Carver and Walker, 1995; Gil, 2002; Hess and Greenberg, 2012; Ding et al., 2021). The importance of exogenous nucleotides for immune function and disease resistance has been demonstrated through studies examining the effects of both nucleotide supplementation and deprivation, particularly in animal models. Insufficient dietary nucleotide availability under stress can impair T-cell-mediated lymphocyte proliferation, antigen presentation by macrophages and dendritic cells, phagocytic activity, antibody production, and cell signalling, compromising immune surveillance (Jyonouchi, et al 1994; Kulkarni et al., 1994). This immune dysfunction may increase susceptibility to disease and mortality. For instance, Kulkarni et al. (1986) and Yamamoto et al. (1994) reported reduced mortality in nucleotide-fed animals following experimental bacterial challenge compared to nucleotide-deficient conditions. Similarly, Guo et al. (2016) observed improved survival in Pacific shrimp supplemented with nucleotides, identifying a critical threshold of $\geq 90 \text{ mg} \cdot \text{kg}^{-1}$ necessary for the effect to be expressed. Studies by Yaseen et al. (2020), Novriadi et al. (2021), and Chen et al. (2022) found comparable dose-dependent improvements in survival rates in farmed fish, shrimp, and prawns, dosed with higher nucleotide levels outperforming nucleotide-free diets.

Fanslow et al. (1988) demonstrated that mice fed a nucleotide-free diet exhibited reduced survival and increased fungal growth following *Candida albicans* infection compared to nucleotide-supplemented mice. Similar observations have been reported in porcine and avian models, where nucleotide supplementation reduced mortality rates and enhanced disease resistance, particularly under high-stress conditions such as crowded housing or increased disease risk (Li et al., 2015; Androni et al., 2018; Leung et al., 2019; Kamel et al., 2021; S-Yaseen et al., 2020; Yong et al., 2020). Overall, dietary nucleotides appear to enhance resistance to and recovery from experimentally induced bacterial, fungal, and viral challenges,

suggesting improved immune surveillance particularly where housing density is high, stress is high and risk of disease increased (Li et al., 2015; Androni et al., 2018; Leung et al., 2019; Hossain et al., 2020; S-Yassen et al., 2020; Yong et al., 2020; Kamel et al., 2021). In contrast, nucleotide-free diets impair resistance in animal models, highlighting the critical role of dietary nucleotides in supporting immune function (Holen et al., 2005; Li and Gatlin, 2006; Do-Huu, 2016; Mohammed et al., 2020; Vallini et al., 2021; Tie et al., 2021b; Chen et al., 2022).

Available data also suggest that dietary nucleotide supplementation may positively impact survival rates by enhancing both innate and adaptive immune responses, improving cell signalling, and promoting anti-inflammatory cytokine pathways over pro-inflammatory ones (Superchi et al., 2012; Li et al., 2015; Novriadi et al., 2021; Tie et al., 2021). For example, studies by Alizadeh et al. (2016), Wu et al. (2018), and Kamel et al. (2021) demonstrated that nucleotide supplementation enabled chickens to mount a faster and stronger antibody response to vaccine challenge compared to nucleotide-deficient controls. Similar immune enhancement effects have been observed in aquaculture, rodent, and bovine models, where nucleotide supplementation was associated with enhanced gene expression, increased antioxidant enzyme activity, upregulated Toll-like receptors and Toll-interacting protein expression, improved T-cell proliferation, natural killer (NK) cell activity, B-cell activation, immunoglobulin secretion, macrophage activity, phagocytosis, cellular autophagy, and anti-inflammatory cytokine production (Burrells et al., 2001; Cameron et al., 2001; Choudhury et al., 2005; Hess and Greenberg, 2012; Xu et al., 2013; Tie et al., 2021a; Tie et al., 2021b; Dinardo et al., 2022; Maggiolino et al., 2023). In human studies, nucleotide supplementation in infants has been shown to enhance antibody responses to vaccination and improve T-cell function (Yau et al., 2003; Hawkes et al., 2006). Additionally, Navarro et al. (1999) reported that the inclusion of nucleotides in milk formula for preterm infants increased immunoglobulin levels and lymphocyte activity, further supporting the role of nucleotides in improving immune function.

Several studies have investigated the effects of nucleotide supplementation on *in vivo* and *in vitro* immune function in recreational athletes subjected to high-intensity exercise stress. Mc Naughton et al. (2006), and Mc Naughton et al. (2007) reported transient improvements in secretory IgA mucosal immunity and attenuation of cortisol release immediately following high-intensity and more prolonged exercise. In contrast, Riera et al. (2013) observed no significant changes in secretory IgA secretion after a submaximal cycling test to exhaustion but corroborated findings of reduced cortisol release. Other studies have examined the impact

of nucleotide supplementation on basic immune cell enumeration (e.g., lymphocytes, neutrophils, basophils) but found no evidence of immune-enhancing effects (Ostojic et al., 2013; Sterczala et al., 2016). However, functional improvements in adaptive immune system biomarkers, such as increased lymphocyte proliferation capacity and enhanced natural killer cell cytotoxic activity, have been reported following acute exercise (Ostojic et al., 2013; Riera et al., 2013; Casajus et al., 2014). Collectively, these limited data suggest a potential role for dietary nucleotide supplementation in supporting human immune function and attenuating activation of the hypothalamic-pituitary-adrenal (HPA) axis following exercise. Nonetheless, further research is required to clarify the extent and mechanisms by which dietary nucleotides influence immune responses under exercise-induced stress.

Several methodological approaches have been adopted to quantify both the innate and adaptive immune system responses to exercise (Simpson et al., 2015). Predominately, a range of in vitro techniques such as cell enumeration (cell counting and identification) as well as functional activity assays which provide precise, controlled assessments of immune cell populations and their responses (Albers et al., 2005; Albers et al., 2015). Although in vitro methods enable rigorous control over cellular, chemical, and physical environments, precise time point assessments they fail to replicate the complex, integrated in vivo interactions required for immune function in exercising human and animal models. The immune system operates as a coordinated network, making isolated in vitro assessments of specific immune components less representative of the dynamic orchestration observed in vivo (Albers et al., 2015). Consequently, in vitro approaches have been criticised, on their ‘snapshot’ isolation, leading to a greater emphasis on in vivo models that better capture the integrated immune response during and after exercise (Albers et al., 2005; Roger et al., 2008; Albers et al., 2015; Bermon et al., 2017; Simpson et al., 2020; Shamsi et al., 2023). In vivo models offer critical insights into the immune system's integrated responses, often evaluated through observable outcomes such as tissue inflammation and oedema (Roger et al., 2008; Bermon et al., 2017). They also establish direct associations between immune responses and clinical outcomes, such as disease progression and recovery (Strickler et al., 1995; Zaman et al., 1997; Levis et al., 2006; Mose et al., 2017; Perkins and Davison, 2021).

Typically, these in vivo approaches adopt several broad methodological approaches regarding T-cell-mediated immune responses. Studies have used vaccine challenge methods, whereby participants are exposed to a novel vaccine delivered systemically either before or after stressor

application in animal and human models (Pascoe et al., 2014; Campbell and Turner et al., 2018; Chastin et al., 2021; Dinas et al., 2022) with the outcome of viral antibody titres production or cell functional responses assessed to reflect both the induction and integration of immune memory. Another widely used approach is the delayed-type hypersensitivity (DTH) test, which involves intradermal antigen injection (likely to previously be encountered) and subsequent assessment of skin inflammation reaction(s) (e.g., erythema and oedema) ~48 hours later (Bruunsgaard, et al., 1997; Simpson et al., 2015). While informative, DTH tests are antigen specific and thus limited by the participant's prior immune exposure and embedded memory to the specific antigen in question (Lyerly, 2003). Contact hypersensitivity (CHS), a subtype of DTH, involves skin reactions to novel chemicals or allergens and is used as a simple in vivo assay to evaluate cell-mediated immune responses (Friedman et al., 2007; Frew et al., 2021). Sensitisation (induction) occurs when epidermal and dermal cells are topically exposed to an exogenous haptens, while the subsequent elicitation phase or reassessment of immunological memory manifests as a measurable delayed hypersensitivity response (e.g., skin erythema and oedema) at a secondary skin site re-exposed to the antigen (Friedmann, 2007; Harper-Adams et al., 2011; Erkes and Selvan, 2014; Frew et al., 2021). Diphenylcyclopropenone (DPCP), a potent sensitizer, is widely used in experimental and clinical CHS studies to induce and investigate cell-mediated immunity (Friedman, 2007; Diment et al., 2015; Ghandi et al., 2021).

Several studies have employed DPCP-induced CHS to evaluate the effects of exercise, psychological stress, and nutritional interventions on in vivo immune responses (Harper-Smith et al., 2011; Diment et al., 2015; Davidson et al., 2015; Jones et al., 2017; Edwards et al., 2018). DPCP-induced CHS has been validated as a reliable marker of exercise-induced cell-mediated immunity (Friedman et al., 2007; Harper-Smith et al., 2011), making it a novel tool for assessing the effects of dietary nucleotide supplementation on integrated T cell mediated immunity following intense exercise. The aim of this study was to examine the effects of 30 days purine and pyrimidine supplementation upon DPCP immune response following high intensity repeated sprint activity. It is hypothesised that dietary nucleotide supplementation would enhance the induction and elicitation phases of T-cell mediated immunity compared to the placebo conditions.

4.3 Methods

4.3.1 Participants

Twenty-four recreationally active male participants (Table 4.0) volunteered to participate and undertook a double-blind matched pairs experimental design across all conditions in a fasted state. All were non-smokers and free from any known illness or injury as ascertained by a Readiness to Exercise Questionnaire. All participants were fully informed verbally and in writing about the nature and discomforts of this study, including all potential risks before giving their consent to participate. The studies were approved by the Research Ethics Committee of Liverpool John Moores University.

Table 4.0. Descriptive characteristics of participants displaying means $\pm$ SD for age (years); body mass (kg); height (cm) and $\dot{V}O_{2\max}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$). ($n=24$).

Variables	Participants		
	Combined	Placebo ($n=9$)	Nucleotide ($n=15$)
Age (years)	22.7 ± 3.7	22.7 ± 2.5	23.2 ± 2.8
Body Mass (kg)	74.4 ± 7.6	72.6 ± 7.1	76.2 ± 6.9
Height (cm)	177.7 ± 5.6	177.7 ± 5.3	178.4 ± 7.0
$\dot{V}O_{2\max}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	49.0 ± 5.1	49.1 ± 5.8	49.2 ± 5.6

4.3.2 Experimental Protocols and Designs

Participants were required to attend the lab on 6 separate visits of varying duration firstly, for an initial $\dot{V}O_{2\max}$ fitness assessment via a 20-meter shuttle test (Ramsbottom, 1995) was performed and used to facilitate participant allocation to respective nucleotide or s experimental conditions upon the basis of aerobic capacity. This was then followed by familiarisation of RAST performance tests and collection of baseline measures for uric acid. Participants were asked refrain from exercise, and alcohol for 24 hours prior to all physiological assessment which was verified by verbal confirmation on subsequent visits. All assessments were undertaken in an overnight fasted state. All participants then undertook a baseline anthropometry and familiarisation performance test consisting modified repeated anaerobic sprint test. Upon completion of RAST familiarisation the participants were then allocated to the experimental conditions using a matched pairs design minimisation of difference protocol following the process of Treasure and Mc Crea (1998) and Treasure and Farewell (2012) to a

30-day nucleotide (n=15) or placebo ingestion period (N= 9), ingesting either nucleotide (Nutritide; Pro-bio, Daresbury, UK) derived from yeast (*Saccharomyces cerevisiae*) at the designated loading doses of 2520 mg or a maltodextrin and binding agents' placebo in anonymised capsules. The dosing regimen consisted of 6 capsules x 3 times per day after meals. Upon completion of the initial 30-day experimental supplementation phase participants returned for RAST performance (4 sets of 6 x 35m sprints with a recovery period of 5 minute separating each set. Each participant completed a total of 24 sprints (4 sets X 6 sprints sets) and in vivo immune function induction, sensitisation, and assessment. After each sprint, heart rate (HR) (Polar, Kempele, Finland), Ratings of Perceived exertion (RPE) (Borg, 1970; Scale 6-20), Sprint Times (s) (ST) were recorded. Fatigue index was derived following the procedures of Fitzgerald et al. (1993). A 10 mL venous blood samples were drawn to determine circulating cortisol and uric acid levels at rest and following exercise performance in line with post exercise, 2 hrs and 24 hours after test completion. A finger prick blood sample (10ul) for blood Lactate (La) was taken by a safety lancet (Sarstedt, Germany) and analysed by a lactate pro analyser (Lactate Pro, Arkray, Japan) at rest and after each sprint set.

Briefly, for immune function assessment participants had an absorbent patch attached to their lower back with the chemical diphenylcyclopropanone (DPCP) impregnated into it and remained in place for 48 hours to induce sensitisation and in vivo immune memory to the DPCP agent and then discarded by the subject. Twenty-eight days later participants returned and had eight similar patches attached to the medial aspect of the upper arm with varying doses of a control and DPCP agent to determine a dose response effect for the effectiveness of immune memory recall (elicitation). The elicitation patches remained in place for 6 hours and was then removed. Two days later (48 hours later) participants returned to have the sites of patch application assessed for the outcome variables of tissue erythema (redness) oedema (swelling) and area of response. No supplements were consumed on the day of assessment. Supplement, compliance was determined by returned capsule count. Minimum requirement was for all participants ingest at least 80% of allocated dose as an arbitrary cut off for supplementation compliance. Where they fell below this rate (<80%) (432 capsules) or could not verify consumption of the supplement they were excluded from the study.

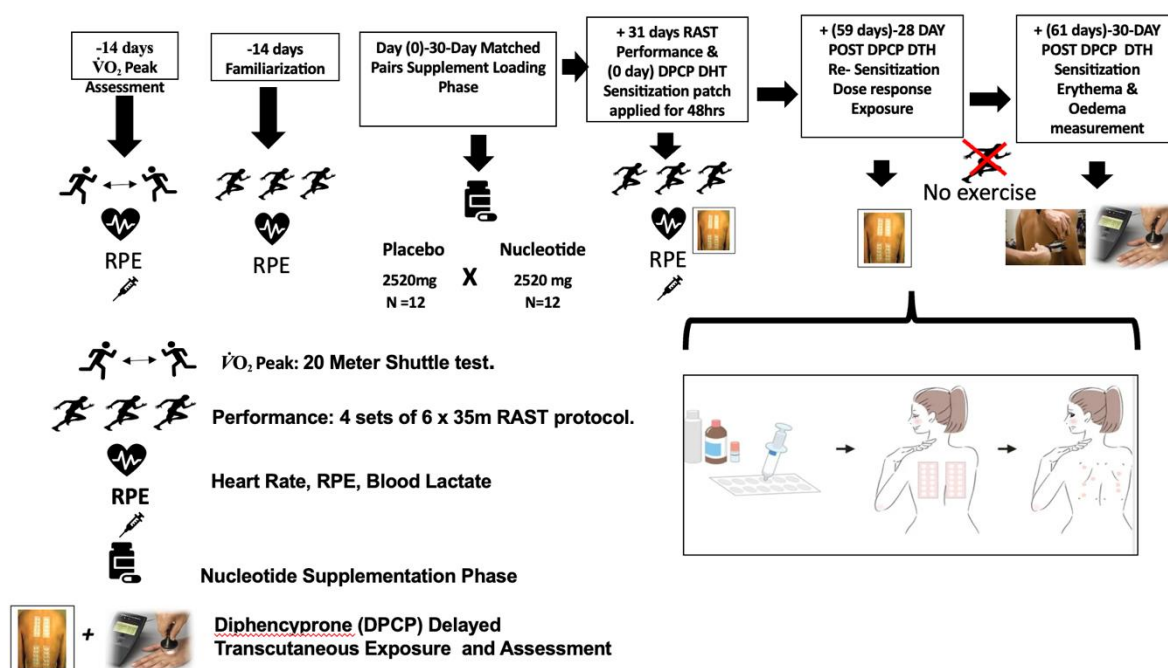


Figure 4.1.1. Experimental design schematic for supplementation and in vivo DPCP immune assessment.

4.3.3 Anthropometry: Body Mass and Height

Following the provision of informed consent, each participant underwent assessment of anthropometry during the first visit of their respective trials. Using a stadiometer (SECA 213, Cranlea and Co, Bournville, Birmingham, UK), height was measured from the vertex of the cranium to the plantar surface of the foot and recorded in centimetres (cm) to one decimal point. Body mass (kg) was also recorded using digital column scales (SECA 704, SECA, UK). The procedures followed the guidelines of the International Society for Kinanthropometry guidelines ISAK (Marfell-Jones *et al.*, 2006).

4.3.4 Cardiorespiratory Measurements: Estimated Maximal oxygen uptake (20-meter shuttle test)

All participants completed an incremental exercise 20-meter multistage shuttle (20-MST) run test in the physiology laboratory of the TRB building for the estimation of maximal oxygen uptake (Ramsbottom *et al.*, 1985). Prior to beginning the test, a Polar HR monitor was fitted and recorded throughout. The 20-MST running test commenced at $8.5 \text{ km} \cdot \text{h}^{-1}$ and increased by $0.5 \text{ km} \cdot \text{h}^{-1}$ every 1 minute with participants running between two lines set 20 m apart at a pace dictated by an audio signal emitted at appropriate intervals to replicate the running speed required. The test was terminated when the participant failed twice to reach within 3 m of the

20-MST line in time with consecutive tones (objective evaluation) or the participant felt unable to complete another shuttle at the audio signal designated speed (subjective evaluation). The total number of levels and shuttles covered during the 20-MST (including the last incomplete shuttle) was designated as the testing score and converted to an estimated aerobic capacity using a conversion formula (Paradisis et al., 2014). Laboratory environmental conditions were recorded during all exercise testing procedures and remained constant with a temperature at $17 \pm 6^{\circ}\text{C}$ and relative humidity at $48 \pm 5\%$. Rating of perceived exertion (RPE) were implemented to anchor the perception of effort to be utilised later during the repeated anaerobic sprint test trials (Draper and Whyte, 1997).

4.3.5 Heart Rate

Heart rate (HR) ($\text{b}\cdot\text{min}^{-1}$) was continually monitored and recorded during all experimental trials by a wearable electrocardiography (Polar FT1, Polar UK). In all studies, heart rate ($\text{b}\cdot\text{min}^{-1}$) was continually measured at 5-second intervals; data as presented represents an average over designated time periods. The polar HR chest strap to determine heart electrical activity was worn directly below the chest at $\sim\text{V5}$ level with the transmitter positioned centrally on the xipho-sternum then adjusted to minimise any movement during each exercise trial. Conductive ultrasound probe gel was applied to the electrodes to increase conductivity of the signal and improve reliability of measurement in the early stages of exercise.

4.3.6 Rating of Perceived Exertion (RPE)

RPE was recorded, using the Borg 6-20 (Borg, 1970) scale, at the end of each shuttle stage during the multi-stage maximal oxygen uptake assessment and at the end of each set within the RAST experimental tests. Participants were familiarised using the Borg scale during the initial maximal incremental 20-MST exercise test to volitional exhaustion. Each end of the Borg scale was ‘anchored’ during the 20-MST exercise protocol so as to introduce the perception of effort associated with progression through the scale from 6 [rest] through 20 [maximal effort], (Birk and Birk, 1987). This process provides a reference point against which participants can refer/gauge their effort and subsequent efforts during RAST trials.

Rating	Description
6	No exertion at all
7	Extremely Light
8	
9	Very Light
10	
11	
12	
13	Somewhat Hard
14	
15	Hard
16	
17	Very Hard
18	
19	Extremely Hard
20	Maximal Exertion

Borg scale for reporting subjective rating of Perceived exertion (Borg, 1970).

4.3.7 Collection, Preparation, Storage and Analysis of Blood Samples

Venous blood samples were collected by a qualified phlebotomist from a superficial vein in the ante-cubital fossa of the forearm using standard venepuncture techniques (Vacutainer Systems, Becton, Dickinson). Briefly, a tourniquet was applied to the upper arm to induce an occlusion in venous return; the medial antecubital vein was cleaned with a Medi-swab isopropyl alcohol wipe (Seaton Health Care Group, Oldham, UK). The butterfly needle (BD safety set 21 G $\frac{3}{4}$ Becton Dickinson, Oxford, UK) was inserted, once a flashback was observed, the needle secured, and the tubes were attached. Upon completion of the sampling the needle was removed, and pressure applied to the puncture site to minimise the potential for a subcutaneous haematoma. Blood samples were collected into vacutainers™ of differing volumes (6.0 mL – 10 mL) (Becton Dickinson, Northampton UK). The blood was sampled using a winged infusion set (butterfly needle; Becton Dickinson, Northampton UK). The samples were collected into four vacutainers; a serum separation tube, EDTA and lithium

heparin which was used as a clotting or chelating agent for the blood. Briefly the serum separation tube was allowed to sit at room temperature for approx. 30 minutes until clotted then placed on ice until centrifugation. The samples were then centrifuged (Sigma 3-18 k Scientific Laboratory Supplies, UK) at 1300 x g for 10 minutes at 4°C (Serum and EDTA) or at 1000 X g for 10 minutes at 4°C. The plasma/serum was then carefully removed from the vacutainer using a pipette (Fisherbrand, Finnpipette, U.S.A.) and aliquoted into replicates for future analysis. Following this, plasma and serum were then stored at -80°C (Thermo, ULT Freezer, Ohio U.S.A.).

4.3.8 Capillary Blood Lactate Collection and Analysis

Capillary blood lactate was determined from a finger puncture test. Briefly at the end of each exercise set a finger (1st- 3rd digit) was cleaned with an alcohol swab to remove any sweat or blood and then the skin was penetrated with a sterile single-use lancet (Sarstedt, Germany). Approximately 10 µL of blood was withdrawn via gentle squeezing action upon the finger. Test strips were inserted into a Lactate pro (Arkray, Kyoto, Japan) device which were then placed against the surface of this blood sample until beeps were audible, signifying the start of analysis. Section 2.3.8 sets out the broad assay principle. The same investigator collected and analysed all blood samples throughout the study.

4.3.9 Repeated Anaerobic Sprint Test Exercise Protocol

A high intensity intermittent exercise sprint protocol was performed by each participant. The protocol a modified version of the Draper and Whyte (1997) Repeated Anaerobic Sprint Test (RAST) consisted of 4 sets of 6 x 35 m sprints, with 15 second active run recovery to the commencement start after each sprint and a 5-minute passive recovery separating each set (Figure 4.1.2). Briefly, the test commenced with the participant undertaking a maximal exertion 35-meter sprint with participant then running back (sub-maximal recovery) to the start line before commencing the next sprint with a time limit of 15 seconds between each sprint. Each sprint was automatically timed (Brower Timing System, USA), and HR, and RPE (Borg Scale 6-20), were recorded after each sprint. Blood lactate was recorded prior to exercise commencement (baseline) after each set of 6 X 35m sprints and 5 minutes post protocol completion. Fatigue index (FI) was determined using the percentage decrement calculation (Fitzsimmons et al., 1993):

$$\text{Fatigue} = (100 \times [\text{total sprint time} / \text{ideal sprint time}]) - 100$$

Total sprint time = sum of sprint time from all sprints.

Ideal time = number of sprint x fastest sprint time.

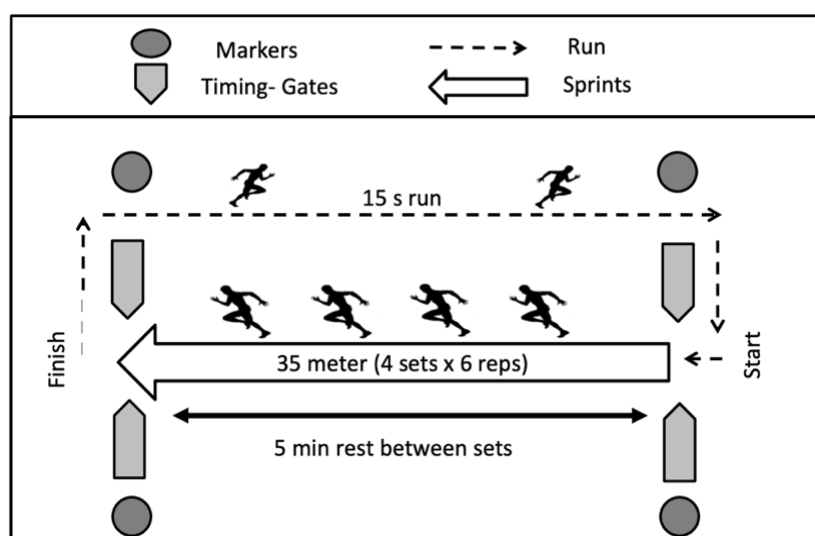


Figure 4.1.2. Protocol schematic for Repeated Anaerobic Sprint Test (Modified Draper and Whyte, 1997).

4.3.10 Dietary Nucleotide Supplementation

Across the studies participants ingested either a capsule consisting of an outer gelatine shell with either 5'-ribonucleotides/RNA nucleotides (Nutri-tide; Pro-bio, Daresbury, UK) derived from yeast (*Saccharomyces cerevisiae*) at the designated loading doses of 2520mg or a maltodextrin (250 mg) and binding agents' placebo in anonymised capsules 6 capsules x 3 times per day after meals (540 in total). The nucleotide mix was a proprietary mix of purine and pyrimidine nucleotides including the following cytosine, uridine, thymidine, adenosine, and guanine. The relative proportions were proprietary and not designated.

4.3.11 Contact-Hypersensitivity: T-cell Mediated In vivo

To examine the influence of nucleotides on in vivo immune function, a simple method called the contact-hypersensitivity skin patch test to a novel antigen diphenylcyclopropanone (DPCP) was undertaken by all participants upon the completion of exercise. Participants were then sensitised to DPCP to examine how the ability of the immune system to remember and mount a response to a haptenogenic agent it perceives as non-self to the body. This is a clinically relevant measure of the induction and immune memory recall of T cell-mediated immunity (Palmer and Freidman, 2004; Freidman, 2007).

4.3.12 Diphenylcyclopropenone (DPCP) [2, 3-Diphenylcycloprop-2-en-1-one]

Diphencyprone (DCP), also known as Diphenylcyclopropenone (DPCP), is a powdery crystal, made into a solution with acetone as the carrier vehicle in this study (Figure 4.1.3). It is most often used in the treatment of alopecia areata and warts. Its primary role is to induce a contact hypersensitivity reaction at the site of administration and induction of an immune memory for future systemic recall upon subsequent re-exposure to the allergen. It is a topical contact immunotherapy agent.

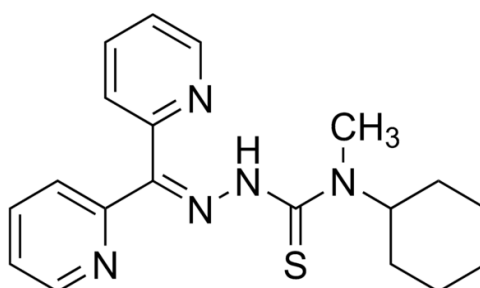


Figure 4.1.3. Chemical arrangement of Diphenylcyclopropenone (DPCP) [2, 3 Diphenylcycloprop-2-en-1-one].

4.3.13 Induction of DPCP Contact Hyper-Sensitivity

Induction of immune sensitisation to the novel immune modulator was facilitated using a single 12-mm aluminium Finn chamber housing an 11-mm scan pour-filter paper patch (Norgeplaster, Alpharma, Norway), soaked (22.8 μL of 0.125% diphenylcyclopropanone (DPCP) in acetone, 30 $\mu\text{g cm}^{-2}$ DPCP) and after a 3–5 minutes drying period applied to the lower dorsal lumbar region and left in place for 48 hours in accordance with previous studies (Harper -Smith et al., 2011; Diment et al., 2015; Jones et al., 2018) (Figure 4.1.4). This application was undertaken 20 minutes post RAST exercise cessation; this was to allow cutaneous blood flow to return toward resting levels in line with previous suggestions (Diment et al., 2015; Jones et al., 2018). The induction of contact sensitivity was carried out on the lower back to ensure no local sensitisation effects on the future elicitation sites and to minimise the potential for Finn chamber dislodgement. Participants were instructed to avoid alcohol and exercise during this 48-hour induction/sensitisation period and avoid anti-inflammatory and steroid medication ingestion. Further, participants were requested not to shower or expose the skin to water until

the 48-hour period had expired to allow optimal sensitisation in line with clinical practice for the immunomodulatory use of DPCP in alopecia (Ghandi et al., 2021).



Figure 4.1.4. Image of 12 mm Finn Chamber ® on Scanpor (Norgeplaster,Alpharma, Norway), used for the induction cutaneous hypersensitivity and elicitation of immune memory after the sensitisation to DPCP.

4.3.14 Elicitation of Immune Memory

Twenty-eight days after the initial DPCP sensitisation phase and induction of immune-memory (sensitisation process), the magnitude of immune memory and responsiveness were quantified by measuring the skin responses elicited by a further secondary exposure to the same DPCP antigen with a dose-response series of DPCP. Briefly, individual Scanpor filter patches were soaked in DPCP following the following dose-response concentration (10 μL of 0.0048%, 1.24 $\mu\text{g cm}^{-2}$; 0.0076%, 1.98 $\mu\text{g cm}^{-2}$; 0.0122%, 3.172 $\mu\text{g cm}^{-2}$; 0.01953%, 5.08 $\mu\text{g cm}^{-2}$; 0.03125%, 8.12 $\mu\text{g cm}^{-2}$; and 0%, 100% acetone) and housed in aluminium Finn Chamber applied to the medial aspect of the upper non-dominant arm. This was fixed to the skin with hypoallergenic tape, previously determined from non-site-specific assessment for the induction of oedema (swelling) and erythema (redness). The DPCP impregnated patches were applied in sequenced order at the upper arm site to minimise the potential of any local anatomical variability influencing responses. The dilution series (Low–High) allowed for quantification of the immune system dose–response curve to DPCP and potential threshold sensitivity determination. The Finn chamber elicitation patches were then removed after exactly 6 hours

of exposure to the skin, disposed of and the skin responses re-assessed at 48 hours after this elicitation exposure in the laboratory via three approaches.

4.3.15 Assessment of Cutaneous Hypersensitivity Response (DTH)

(A) Erythema Response

Elicitation responses to DPCP induction were quantified by the assessment of an objective dermal responses at each measurement site; the erythema (redness) was quantified with an erythema meter (ColorMeter DSMII, Cortex Technology, Hadsund, Denmark) to provide objective assessment of the changes in skin redness (Figure 4.1.5). Briefly, the colorimeter is a narrow-band spectrophotometer, which relies on the different light refraction and spectral curves to determine the absorption of light by haemoglobin (erythema) and melanin (pigmentation). Initially, the DSMII ColorMeter emits a constant light source onto the skin surface within the visible spectrum using two light-emitting diodes (LEDs) (green 568 nm and red 655 nm) that illuminate the skin surface and record the intensity of reflected light using a photodetector (Figure 4.1.5). Erythema is measured by the green and red wavelengths, whereas melanin (pigmentation) is determined by the red wavelengths. The erythema and melanin index values generated reflect that haemoglobin, a primary contributor to skin redness, absorbs light in specific wavelengths, in the range of ~500 to 600 nanometres. As this light interacts with the skin, it undergoes scattering and absorption. The refracted light from the skin is then captured by the colorimeter which converts the light intensity across various wavelengths into corresponding electrical signals. These signals are subsequently processed and analysed using proprietary inbuilt algorithms. By comparing the intensity of light absorbed by haemoglobin/melanin in the skin to a standardised reference, the DSM II ColorMeter quantifies the level of skin redness/pigmentation. The erythema index reported is defined as: $E = 100 \times \log (\text{intensity of reflected red light} / \text{intensity of reflected green light})$ and the melanin index can be defined as: $M = 100 \times \log (1 / \text{intensity of reflected red light})$ wavelengths, whereas melanin (pigmentation) is determined by the red wavelengths. Erythema measured by the DSM II ColorMeter presents with an ICC of 0.84 (van der Wal et al., 2013). To determine the erythema (redness) response, the value from the acetone-only site was subtracted from each elicitation site. The values for all sites were summed to be representative of the overall erythema reactivity of each participant to DPCP in line with previous recommendations (Palmer and Friedmann 2004; Harper-Adams et al., 2011; Diment et al., 2015; Jones et al., 2017).



Figure 4.1.5. Image of the DSM II ColorMeter II for the assessment of skin erythema (redness) and pigmentation (Melanin).

(B) Tissue Oedema (Skinfold Thickness)

Assessment of contact hypersensitivity (oedema) responses were further determined from triplicate measurements of skin-fold thickness taken at each exposure site using a modified skinfold calliper i.e., the spring was removed (Harpenden Skin-fold Calliper, British Indicators, England). Skin-fold thickness was recorded to the nearest 0.1 mm by the investigator by placing the jaws of the calliper at the outer diameter of the visually obvious skin response site and measuring skin thickness only (no subcutaneous fat). This anthropometric approach has previously been utilised and provides an objective measure of skin oedema (inflammatory swelling) at the site of elicitation (Figure 4.1.6) (Harper-Adams et al., 2011; Diment et al., 2015; Jones et al., 2017). The values for all sites were summed to be representative of the overall oedema reactivity of each participant to DPCP in line with previous recommendations (Palmer and Friedmann 2004; Harper-Adams et al., 2011; Diment et al., 2015; Jones et al., 2017). The increase in skin thickness over the 48-hr period was calculated for each challenge site by subtracting the acetone (control) skinfold thickness from the 48-hr post challenge thickness. The "mm increase sum" is derived from the addition of the increases in skin thickness of each of the dose response concentrations for each DPCP and allows the entire dose-response curve to be approximated as a single value. We noted oedematous vesicular changes across some but not all participants, particularly at higher dose (Figure 4.1.6).

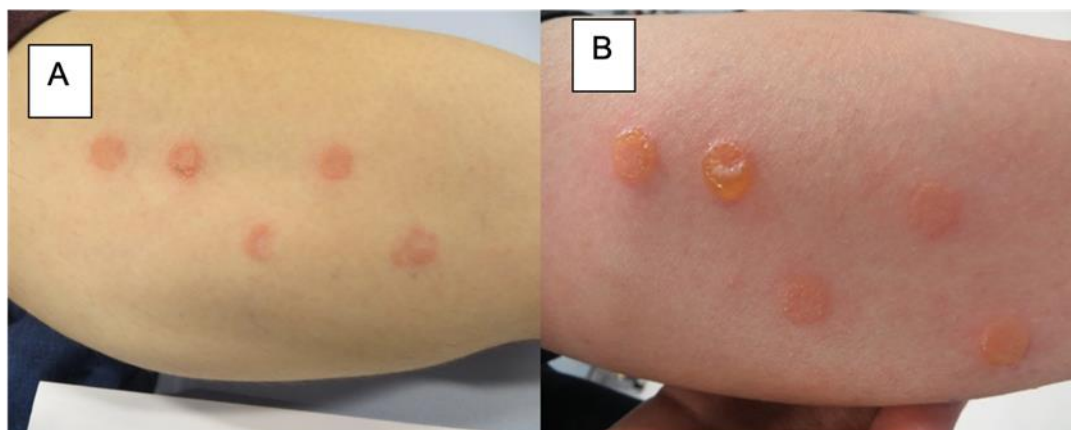


Figure 4.1.6. Example image of participant's arm 48-hours after the sensitisation dose response to DPCP, image A elicitation with placebo while image B presents elicitation with nucleotide supplementation. Note: the response variation in term of erythema, oedema, and vesicle formation.

(C) Total Cutaneous Hypersensitivity Response Area

Across each experimental condition the area of total dermal response to DPCP exposure under each treatment dose was determined as an index of dermal reaction. Following elicitation, a digital image of each dose response series of Finn-chamber exposure was captured 48 hours after exposure. The purpose of this assessment was to capture changes in skin erythema and oedema/pigmentation across the dose response range. This allowed for assessment of the area of response. Previous observation had noted that responses not only included redness and oedema, but the size of response area differed across individuals with the diameter much greater in some than others. Observation from previous trials had noted wide inter and intra individual responses whereby changes in erythema and oedema were minimal (low responder) and other had significant reactions (high responders) with erythema, oedema, and vesicle formation (Figure 4.1.6). To objectively quantify this range of response from barely discernible to strong reaction a digital image was captured, and this image was then entered into Image J (National Institutes of Health, Maryland, USA), inverted and the area of each dermal response subjectively assessed. This involved identifying the subjective boundary of each response area and determining the total pixilation present within that area. The sum of the total area (Σ total area) - area for the acetone response was quantified and reported in absolute image J pixilation area values (Figure 4.1.7).

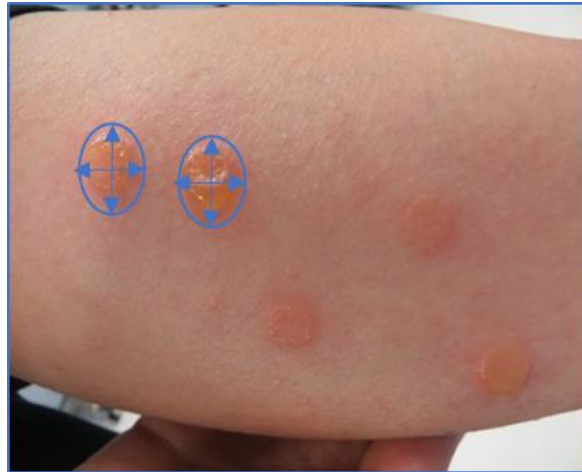


Figure 4.1.7. Example image of participant's arm 48-hours after the sensitisation dose response to DPCP, image indicates the determination of Total Cutaneous Hypersensitivity Response Area.

4.3.16 Statistical Analysis

Statistical analysis was carried out using the Statistical package for Social Science software program (SPSS V. 23, for Windows, Chicago, Illinois, USA). All Data was presented as means ($\pm$ SD). Data was assessed for normality of distribution. An independent sample T Test and two-way mixed ANOVA, within subjects factor was set to measure any significant outcome variable of tissue oedema and erythema. Two-way within subject ANOVA 'condition' and 'time' was applied to the above parameters. To correct any violations of sphericity the degrees of freedom were corrected using Greenhouse-Geiser epsilon (<0.75) or Huynh-Feldt epsilon (≥ 0.75), as applicable (Field, 2000). Post-hoc Tukey analysis was used to detect difference where a main effect was apparent. Statistical significance was set at $p \leq 0.05$ for all tests.

4.4 Results

4.4.1 Repeated Anaerobic Sprint Test Performance

Following a matched pairs design, participants successfully completed the 4 sets of 6 x 35m high intensity intermittent exercise sprint protocol, after 30-days of nucleotide or placebo supplementation. Average sprint times across sets post supplementation are presented (Table 4.1.0). There was no significant effect of nucleotide supplementation upon sprint performance following either placebo or nucleotide supplementation ($F_{1.3, 29.1} = 0.28$, $P = 0.66$). In addition, there was no significant interactions between conditions ($P \geq 0.05$).

Table 4.1.0. Sprint performance times (s) following different supplementation condition. No significant effects of supplementation were evident. Data are expressed as Mean $\pm$ SD.

Sprint	Condition		<i>P</i> value
	Placebo (S)	Nucleotide (S)	
Set 1	5.93 $\pm$ 0.43	6.35 $\pm$ 0.89	$p \geq 0.05$
Set 2	6.11 $\pm$ 0.52	6.38 $\pm$ 0.94	$p \geq 0.05$
Set 3	6.31 $\pm$ 0.57	6.63 $\pm$ 1.47	$p \geq 0.05$
Set 4	6.41 $\pm$ 0.59	6.66 $\pm$ 1.22	$p \geq 0.05$

4.4.2 Fatigue Index Data

Following the placebo and nucleotide supplementation phase, there was no significant effect upon the fatigue index (%) expressed during sprint performance ($t_{(22)} = -0.81$, $P = 0.43$).

Table 4.1.1. Average fatigue index responses(s) (%) for all participant's across all 24 sprints after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Group	Percentage of decrement (%)	<i>P</i> value
Placebo	17.6 $\pm$ 6.1	$p \geq 0.05$
Nucleotide	21.2 $\pm$ 14.1	$p \geq 0.05$

4.4.3 Heart Rate Data

The mean heart rate for all 4-set combined, after 30-days placebo loading were $170 \pm 12 \text{ b} \cdot \text{min}^{-1}$ and 30-days nucleotide loading were $162 \pm 14 \text{ b} \cdot \text{min}^{-1}$. There was no significant main effect or interaction effect of supplementation condition upon heart rate responses during sprint performance ($F_{1.8, 38.5} = 0.23$, $P = 0.77$).

Table 4.1.2. Average heart rate response(s) ($\text{b} \cdot \text{min}^{-1}$) for all participants after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Sprint	Condition		<i>P</i> value
	Placebo	Nucleotide	
Set 1	165 ± 10	156 ± 15	$p \geq 0.05$
Set 2	170 ± 12	163 ± 15	$p \geq 0.05$
Set 3	172 ± 12	164 ± 14	$p \geq 0.05$
Set 4	171 ± 13	166 ± 12	$p \geq 0.05$

4.4.4 Rating of Perceived Exertion (RPE) Data

The mean RPE for all 4-sets of repeated sprint activity combined, after 30-days placebo loading were 14 ± 2 and 30-days nucleotide loading were 14 ± 2 . There was no significant main or interaction effect of nucleotide supplementation upon the RPE (Borg Scale) during sprint performance ($F_{1.5, 32.1} = 0.31$, $P = 0.67$).

Table 4.1.3. Average RPE responses(s) (AU) for all participants after placebo and nucleotide supplementation. Data are expressed as Mean $\pm$ SD.

Sprint	Condition		<i>P</i> value
	Placebo	Nucleotide	
Set 1	12 ± 2	12 ± 3	$p \geq 0.05$
Set 2	14 ± 1	14 ± 3	$p \geq 0.05$
Set 3	15 ± 2	15 ± 3	$p \geq 0.05$
Set 4	16 ± 1	16 ± 2	$p \geq 0.05$

4.4.5 Blood Lactate Data

The mean blood lactate for all 4-sets combined following placebo and nucleotide loading for 30 days are presented in Table 4.1.4. There was no significant difference in blood lactate accumulation following RAST exercise performance pre and post supplementation condition ($P \geq 0.05$).

Table 4.1.4. Average blood lactate responses(s) $\text{mmol} \cdot \text{L}^{-1}$ for participants after placebo and nucleotide supplementation pre and post RAST completion. Data are expressed as Mean $\pm$ SD.

Group	Condition		P value
	Pre-test	Post-test	
Placebo	1.69 ± 0.96	10.63 ± 2.23	$p \geq 0.05$
Nucleotide	1.53 ± 0.42	11.24 ± 2.16	$p \geq 0.05$

4.4.6 Uric Acid Data

During the pre and post placebo and nucleotide supplementation phase, there was no significant effect of nucleotide supplementation on uric acid during sprint performance ($F_{2.7, 59.3} = 4.03$, $P = 0.01$). In addition, there was no significant interaction between conditions ($P \geq 0.05$).

Table 4.1.5. Average uric acid (concentrations) for participants after placebo and nucleotide supplementation for 30 days pre and post RAST completion. Data are expressed as Mean $\pm$ SD.

Time Point	Condition		P value
	Placebo	Nucleotide	
Pre exercise	203.15 ± 78.19	320.86 ± 66.14	$p \geq 0.05$
Post exercise	251.50 ± 50.36	346.64 ± 62.82	$p \geq 0.05$
Post 90 minute	302.59 ± 95.86	408.18 ± 96.61	$p \geq 0.05$
Post 2 hour	259.04 ± 126.51	409.72 ± 109.62	$p \geq 0.05$
Post 24 hour	271.75 ± 79.40	294.41 ± 70.79	$p \geq 0.05$

4.4.7 Serum Cortisol Data

Serum cortisol release increased significantly pre-to post RAST exercise returning toward baseline at 2 hours post activity ($P \leq 0.05$). No significant interaction effects or difference between supplementation conditions were observed at all time points ($P \geq 0.05$).

Table 4.1.6. Serum cortisol ($\text{ng} \cdot \text{mL}^{-1}$) responses prior to and following the performance of the high intensity RAST protocol grouped by experimental treatment. Data are expressed as Mean $\pm$ SD.

Time Point	Condition		P value
	Placebo	Nucleotide	
Pre exercise	140.1 $\pm$ 39.9	144.18 $\pm$ 31.92	$p \geq 0.05$
Post exercise	182.5 $\pm$ 48.6	198.65 $\pm$ 67.56	$p \geq 0.05$
Post 90 minute	142.4 $\pm$ 50.0	151.99 $\pm$ 61.48	$p \geq 0.05$
Post 2 hour	121.1 $\pm$ 42.0	134.35 $\pm$ 59.62	$p \geq 0.05$
Post 24 hour	135.1 $\pm$ 46.4	152.86 $\pm$ 39.20	$p \geq 0.05$

4.4.8 Erythema and Melanin Response

Upon completion of the final exercise stress protocol, all participants underwent the assessment of immune function using the Contact-hypersensitivity Skin Patch Test. There was no significant effect of nucleotide supplementation upon erythema index ($t_{(22)} = 1.02$, $P = 0.32$) and Melanin index ($t_{(22)} = -0.44$, $P = 0.66$) (Table 4.1.7).

Table 4.1.7. Average DTH responses following 48 hours post-elicitation of DTH response with DPCP grouped by experimental treatment. Columns data reflect the means of summed responses (Σ) of the five doses of DPCP. Data are expressed as Mean $\pm$ SD.

Group	Erythema Index	Melanin Index	P value
Placebo	14.72 $\pm$ 3.63	36.11 $\pm$ 4.40	$p \geq 0.05$
Nucleotide	13.20 $\pm$ 3.61	36.85 $\pm$ 3.82	$p \geq 0.05$

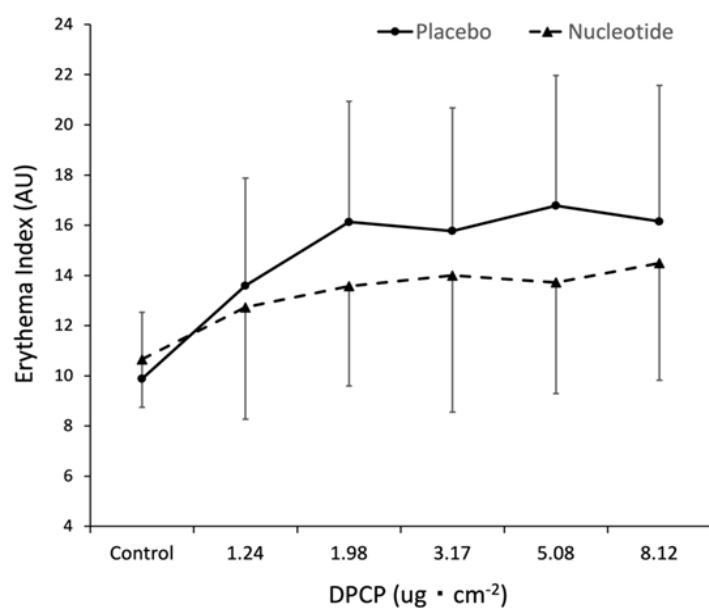


Figure 4.1.8. DTH responses following 48 hours post-elicitation with DPCP at different doses grouped by experimental treatment. Data are expressed as Mean ± SD.

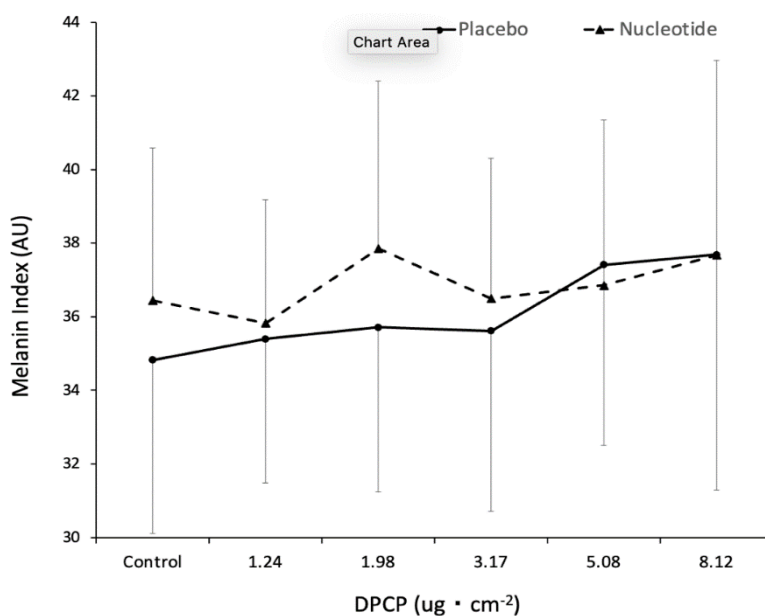


Figure 4.1.9. DTH responses following 48 hours post-elicitation with DPCP at different doses grouped by experimental treatment. Data are expressed as Mean ± SD.

4.4.9 Total Redness Area

There was no significant effect of nucleotide supplementation upon the area of pixelation redness score ($t_{(22)} = 1.87$, $P = 0.07$).

Table 4.1.8. Average tissue redness index responses 48 hours post-elicitation of DTH response with DPCP grouped by experimental treatment. Column data reflect the means of summed area of redness responses (Σ) for the five doses of DPCP. Data are expressed as Mean $\pm$ SD.

Group	Redness Score	<i>P</i> value
Placebo	29014 $\pm$ 31024	$p \geq 0.05$
Nucleotide	25505 $\pm$ 37504	$p \geq 0.05$

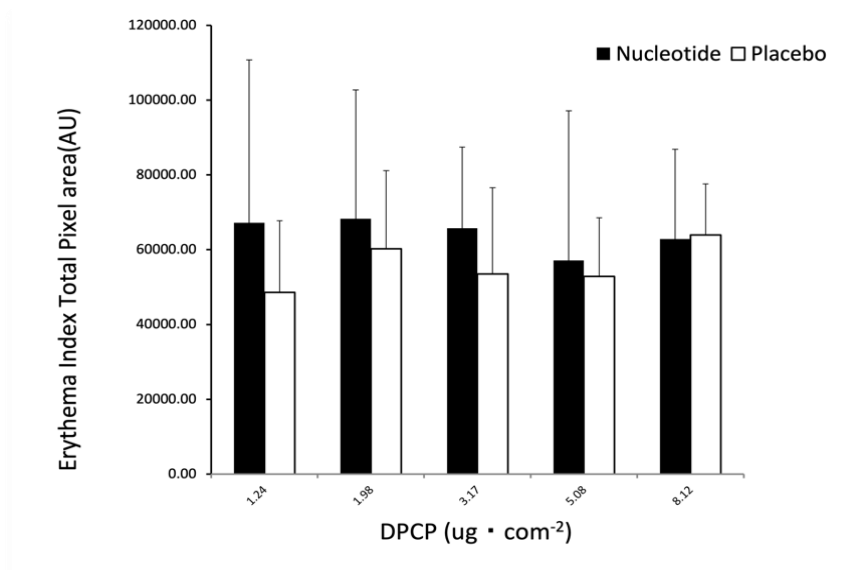


Figure 4.2.0. DTH responses following 48 hours post-elicitation with DPCP at different doses grouped by experimental treatment. Data are expressed as Mean $\pm$ SD.

4.4.10 Tissue Oedema Response (Skinfold Thickness)

Tissue oedema responses (mm) to dose response DTH exposure are presented in Table 4.1.9. No significant differences in oedema were present ($P \geq 0.05$).

Table 4.1.9. Average tissue oedema (mm) responses change following 48 hours post-elicitation of DTH response with DPCP grouped by experimental treatment. Column data reflect the means of summed responses (Σ) of the five doses of DPCP. Data are expressed as Mean $\pm$ SD.

Group	Thickness Score	<i>P</i> value
Placebo	0.27 ± 2.97	$p \geq 0.05$
Nucleotide	0.65 ± 1.48	$p \geq 0.05$

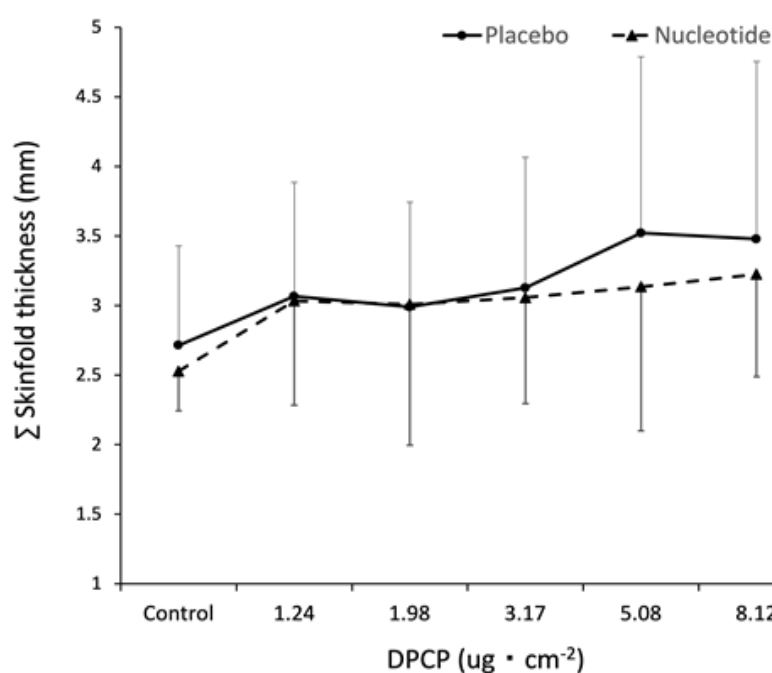


Figure 4.2.1. DTH tissue oedema responses 48 hours post-elicitation with DPCP at different doses grouped by experimental treatment. Data are expressed as Mean $\pm$ SD.

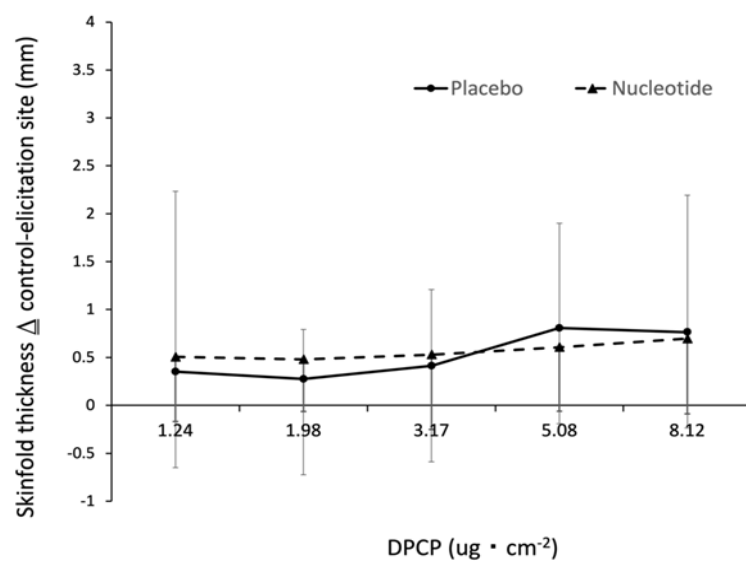


Figure 4.2.2. DTH changes in tissue oedema responses 48 hours post-elicitation with DPCP at different doses grouped by experimental treatment. Control minus (-) elicitation site skin-thickness. Data are expressed as Mean $\pm$ SD.

4.5 Discussion

The aim of this study was to investigate the effects of 30 days of purine and pyrimidine nucleotide supplementation (~2520 mg per day) compared to a placebo on cutaneous hypersensitivity immune responses (CHS) to DPCP following an acute bout of high-intensity repeated sprint activity. Previous research has demonstrated that exercise performed prior to sensitisation with DPCP can impair the establishment of T-cell-mediated immunity. The principal findings of this study indicate that, no significant differences on in vivo CHS immune responses or cortisol levels were observed in recreationally active males following chronic nucleotide supplementation. These findings suggest that a 30-day dietary nucleotide supplementation regimen does not significantly enhance in vivo immune responses or hypothalamic-pituitary-adrenal (HPA) axis activation in response to acute high-intensity repeated sprint exercise.

In this chapter immune function was determined through an in vivo immune approach. Contact hypersensitivity (CHS) is an immune-mediated skin reaction triggered by an initial exposure to novel chemicals and/or allergens (haptens) and clinically manifests in the occurrence of a variety of skin conditions (Friedmann, 2007; Karlberg et al., 2008; Schmidt et al., 2016; Ghandi et al., 2021). In the present study, Diphenylcyclopropanone (DPCP), a low molecular weight (<210Da) hapten was used to elicit a type IV delayed-type hypersensitivity inflammatory response mediated by effector T-cell (Harper smith 2011; Diment et al., 2015). This approach offers an integrated evaluation of in vivo immune processes, enabling the assessment of how external stressors influence the establishment of integrated immune function and its capacity to mount future responses to similar challenges (Field, 1996; Albers et al., 2005; Albers et al., 2013). The magnitude of subsequent CHS immune responses to DPCP reflects the status of the immune system during the initial exposure hapten challenge (Friedman, 2006; Martin et al., 2011; Diment et al., 2015). The use of this in vivo model to determine the effect of dietary nucleotides in humans is novel, with limited research exploring the impact of nutritional interventions on exercise-mediated immune responses using this method (Davison et al., 2015; Jones et al., 2107). This approach provides a practical evaluation of the potential impact of nutritional interventions on complex immune biological systems subjected to exercise-induced stress.

The CHS immune response involves two phases: (1) an induction/sensitisation phase, during which the hapten is first applied to the skin, and (2) an elicitation phase, where the hapten is re-applied to a different skin site to evaluate the strength of local and systemic immune response generated from the initial exposure (Friedmann, 2007; Honda et al., 2013; Diment et al., 2015). These phases encompass multiple stages of integrated immune activity: an initial sensitisation phase, a priming and development of effector T-cell response phase, a resolution and induction of immunological memory, phase. In the present challenge model, the induction of CHS i.e., sensitisation to DPCP occurred with topical skin exposure as described in section 4.3.13. Upon exposure DPCP penetrates the epidermal barrier and forms a hapten (DPCP)-protein complex which is phagocytosed by Langerhans and dendritic antigen-presenting cells (APC) residing in the skin, which are then processed onto Major Histocompatibility Type II receptors and presented on the APC cell membrane. The APCs then migrate to lymph nodes, where they present the DPCP antigen to naïve T cells, initiating sensitisation. Naïve T cells differentiate into DPCP-specific effector T cells within 24–48 hours of initial exposure subsequently these cells migrate to the lymph nodes (Chipinda et al., 2011). Activated effector T cells are primed and migrate from lymph nodes to the skin in response to the release of the chemoattractant (histamine), pro-inflammatory cytokines, and adhesion molecules during initial inflammation (Engeman et al., 2004; Honda et al., 2013; Schmidt et al., 2017). Upon encountering DPCP-modified APCs in the skin, T cells (Effector CD4⁺/CD8⁺) reinforce their activation, releasing cytokines such as interferon-gamma (IFN- γ) and tumour necrosis factor-alpha (TNF- α), which amplify local inflammation, recruit additional immune cells, and lead to localised dermal and epidermal inflammation and damage i.e. a delayed-type IV hypersensitivity response at the exposure site within ~24-72-hours. In the resolution phase, effector immune cells undergo apoptosis or migrate away from the inflamed DPCP exposure site reducing local inflammation and swelling (tissue oedema). Keratinocytes, and fibroblasts in the epidermal layer participate in tissue repair processes, including re-epithelialisation and collagen deposition, restoring skin barrier function (Chipinda et al., 2011; Martin et al., 2011). Finally, in the memory phase, Memory T cells generated during the sensitisation phase increase in number over several weeks in the skin and lymphoid organs, conferring a long-lasting enhanced DPCP memory response, and an ability to rapidly respond to subsequent re-exposure to DPCP with specific effector T cells at sites distant from the initial exposure site (Kabashima, et al., 2019). Present observations confirm the initial induction process showed no or limited skin inflammation, erythema, or tissue oedema, due to the requirement for widespread immunological memory to the agent not having been previously established. It is only upon re-

exposure where the immunological sequence above has been established that the sensitised cellular responses are activated and become observable as tissue oedema and redness.

This integrated immunological approach evaluated the influence of dietary nucleotides on the induction of the above complex CHS immune response following high intensity repeated sprint exercise. This complex response being quantified through the assessment of morphological changes in skin inflammation/oedema (skinfold thickness) and erythema (skin redness) following re-exposure (elicitation) to DPCP. The elicitation response is critical for assessing the efficacy of CHS and determining in this study whether nucleotides enhance immune response development (Friedman, 2007). The strength of the elicitation response depends on the effectiveness of the initial sensitisation stimulus and the establishment of immunological memory in both the skin and lymph nodes (Field, 1996; Friedman, 2007; Albers et al., 2005). The more effectively the initial exposure and sensitisation is acquired the lower the subsequent DPCP dose required to elicit a response (Friedman, 2007; Harper-smith et al., 2011). Factors that disrupt or enhance CHS development, such as physiological or psychological stressors, can alter the immune response by changing the threshold DPCP dose required to induce observable tissue oedema and erythema. This phenomenon is well established following the imposition of both physiological and/or psychological stressors before and after other contact hypersensitivity challenges and in vivo vaccine inoculation to access integrated cellular and antibody response function (Lyerly, 2003; Lowder et al., 2005; Rogers et al., 2008; Kohut et al., 2009; Long et al., 2020; Chastin et al., 2021; Bohn-Goldbaum et al., 2020; Dinas et al., 2022). The use of CHS in this study represents a robust method to evaluate the potential immunomodulatory effects of dietary nucleotides in response to exercise-induced stress.

The evaluation of nutritional interventions on exercise-mediated in vivo CHS immune responses has been very limited (Davison et al., 2015; Jones et al., 2017). This study aimed to determine whether nucleotide supplementation could enhance the induction and consolidation phases of the CHS immune response following high-intensity repeated sprint activity (RSA) exercise. The rationale for this investigation stems from evidence suggesting that dietary nucleotides can become conditionally essential i.e. exogenous provision is required to meet immunological demand under elevated stress conditions. In such cases, they function as modulatory nutrients, supporting the proliferation of lymphoid cells, enhancing antigen presentation, facilitating cell signalling pathways, promoting cytokine production, and aiding cell trafficking (Van Buren et al., 1983; Van Buren et al., 1994; Carver et al., 1994; Gil et al.,

2002; Junger et al., 2016; Cekic and Linden, 2016). Furthermore, high-intensity interval exercise has been shown to induce significant metabolic stress and hormonal changes, often resulting in the suppression of biomarkers of immunity determined in vitro (Souza et al., 2021).

T cells, which play a critical role in adaptive cellular immunity and are essential during the effector stages of CHS induction, are particularly susceptible to reductions in number following repeated sprint exercises compared to resting conditions (Fry et al., 1992; Frisina et al., 1994; Kakanis et al., 2010; Souza et al., 2021). For instance, Tossige-Gomes et al. (2016) demonstrated that performing eight 1-minute sprints at 90–100% peak power, interspersed with 75 seconds of active recovery, significantly impairs post-exercise T-lymphocyte proliferation, a critical factor in establishing CHS induction. Similarly, Krüger et al. (2016) highlighted that high-intensity interval exercise affects T-cell subpopulation recruitment and mobilisation, while also increasing apoptosis, thereby compromising the ability of T cells to respond to antigen stimulation and establish new immunity. This has direct relevance to the present work, as it may impair CHS induction and limit subsequent elicitation. Turner et al. (2016) further reported that performing ten 1-minute bouts at 90% of $\dot{V}O_2$ max with low-intensity recovery between efforts can disrupt cell trafficking to the skin. Such disruptions could impair T-cell migration to the site of DPCP application, especially if the period of impairment overlaps with the critical window for DPCP exposure and consolidation (~6 hours). Moreover, Lee et al. (2023) demonstrated that innate immune cell function and enumeration are impaired for up to three hours after 90 seconds of maximal paddling in elite kayakers, potentially affecting the antigen presentation processes required during the early stages of DPCP sensitisation. Similarly, others have reported that innate immune function, as measured in vitro, is impaired within 30 minutes of sprint interval exercise, potentially impacting antigen presentation and phagocytic function (Davidson, 2011; de Oliveira et al., 2019). Collectively, these findings suggest a potential role for dietary nucleotides as immune-modulatory agents to maintain immune function during RSA-type exercise. In this context, nucleotide supplementation could mitigate the immunosuppressive effects of high-intensity exercise, preserving key processes involved in CHS induction and immune response establishment.

As a surrogate measure for the complex immune induction process, the analysis of morphological changes in the present chapter, revealed no significant effects of nucleotides upon the minimum DPCP dose required to elicit a positive CHS response. This finding indicates that nucleotides did not significantly alter immune sensitivity, as expressed through

tissue oedema, erythema, and total erythema area (swelling/inflammation) to DPCP challenge. Similarly, at each individual DPCP dose exposure site, no significant differences in CHS response were observed between nucleotide and placebo conditions. Collectively, these results suggest that the administration of nucleotides following RSA stress provided no detectable immunological advantage using the current experimental approach.

However, as shown in Table 4.1.9, the nucleotide condition appears to evoke a higher but not significant tissue oedema elicitation response compared to the placebo. This potentially reflects a more effective initial DPCP induction and immune activation process, although this observation is confounded by considerable variability in individual responses. Similar findings have been reported by Jones et al. (2017), who found that approximately 58 days of bovine colostrum supplementation did not significantly affect CHS tissue oedema responses across their reported DPCP dose-response range following a more strenuous protocol of 120 minutes of treadmill running at 60% of $\dot{V}O_2$ max. Nevertheless, Jones et al. (2017) observed that bovine colostrum elicited a twofold increase in sensitivity to DPCP at the lowest DPCP exposure dose, suggesting an enhanced immune response during the induction phase that improved subsequent sensitivity to DPCP. However, this effect was not replicated at other DPCP doses in their protocol, indicating potential artifacts in their data acquisition for this time point. Similarly, Davison et al. (2015) examined the effects of carbohydrate ingestion immediately before a 120-minute treadmill run at 60% of $\dot{V}O_2$ max and observed no significant influence on the DPCP concentration required to elicit a CHS tissue oedema response. Furthermore, no significant effects were observed in the overall dose-response sensitivity curve. Taken together, these findings from the current study, along with those of Jones et al. (2017) and Davison et al. (2015), indicate that the chronic provision of nucleotides or bovine colostrum, as well as acute supplementation with carbohydrates, did not produce any discernible enhancement of CHS in vivo immune responses following exercise-induced stress across a range of exercise intensities and durations.

This absence of significant effects on in vivo immune responses following nutritional interventions is not universally observed. Evidence from animal models demonstrates that exogenous nucleotides play a critical role in maintaining delayed-type cutaneous hypersensitivity (DTH) immune responses. For instance, Adjei et al. (1996) reported that a four-week period of nucleotide supplementation significantly enhanced in vivo DTH responses, as evidenced by increased ear thickness (oedema) in response to contact sensitisation agents.

This improvement was attributed to elevated T-cell proliferation, cytokine secretion, and effector T-cell function. Similarly, Yamauchi et al. (1996) observed an enhanced *in vivo* DTH response to footpad inoculation in mice following a four-week mixed nucleotide diet. The study also highlighted a superior lymphocyte proliferative response in lymph nodes among animals fed a mixed nucleotide diet compared to those receiving a single nucleotide supplement. Furthermore, research by Yamauchi et al. (2002) and Kulkarni et al. (2002) demonstrated that mice subjected to chronic tail suspension stress (24 hours per day over four weeks) exhibited improved *in vivo* DTH responses when provided with a nucleotide-enriched diet compared to a standard diet. These findings underscore two critical points: first, that *in vivo* DTH models, like the CHS model used in the present study, are sensitive to stress-induced changes in immune function and respond to nutritional interventions; and second, that nucleotides act as effective immunomodulatory agents in rodent models, which clearly differentiate them from the present chapters human CHS *in vivo* model to detect changes in immunity. This lack of effect upon *in vivo* immune response following nutritional intervention (s) is not always the case.

The sensitivity of CHS/DTH immune responses to external stressors observed in animals is also reflected in human models, which have demonstrated the ability of CHS to detect immune-related changes following exercise compared to no exercise. Specifically, acute exercise performed prior to CHS skin sensitisation with the novel antigen DPCP been shown to impair the induction of T cell specific antigenic memory. Harper-Smith et al. (2011) and Diment et al. (2015) using a matched pairs design report that 120 min of treadmill running at 60% of $\dot{V}O_2$ max significantly impaired the induction phase and altered the elicitation profile of T cell mediated immunity compared to resting controls. Similarly, Davison et al. (2015) and Jones et al., (2017) observed that 120 minutes of treadmill running at 60% of $\dot{V}O_2$ max prior to primary sensitisation to DPCP impairs CHS immunity. These studies reported reduced skin inflammation, as indicated by decreases in summed tissue oedema responses at all DPCP challenge sites and increases in the minimum DPCP dose required to elicit a positive immune response (threshold) during the recall (elicitation) phase of T-cell-mediated memory, relative to resting controls. These findings suggest that moderate-intensity endurance exercise can impair CHS immune responses, as evidenced by changes in morphological markers such as skinfold thickness and erythema. From a methodological perspective, these studies illustrate that the morphological (e.g., skinfold thickness) and colorimetric (e.g., erythema) components of the CHS approach are sufficiently robust to detect the presence or absence of an immune

response. Notably, they have demonstrated exercise-induced differences in these parameters when exercise intensity and duration are sufficient to disrupt normal homeostasis (Harper-Smith et al., 2011; Diment et al., 2015). However, this approach appears less effective in detecting differences arising from nutritional interventions where the effects may be smaller or absent (Davison et al., 2015; Jones et al., 2017). This raises the possibility that the present human CHS immune model tested in this chapter has a high level of biological redundancy, whereby the RSA acute exercise stress applied does not exceed the endogenous immunological homeostatic capacity of the individual participants, thus negating the need for external nutritional support.

The endurance exercise models shown to alter *in vivo* immune responses (Jones et al., 2017 and Diment et al., 2015) differ significantly from the exercise protocol utilised in the present study. The repeated sprint-ability (RSA) protocol involved short (~5–6 seconds) supramaximal efforts, followed by ~15 seconds of light submaximal running, with subsequent sprint efforts. Each set consisted of ~30 seconds of supramaximal effort and ~90 seconds of submaximal recovery (1:3 work-rest ratio), with 5 minutes of rest between sets, amounting to approximately 8 minutes of total physical work time. These characteristics [short but intense bouts of exercise, active submaximal recovery, and extended passive recovery periods] appear insufficient to significantly disrupt normal immunological “reserve capacity” in this *in vivo* assessment model (Pershin et al., 1988; Kassil et al., 1988). Supportive this view, Diment et al., 2015) reported that ≤30 mins of continuous treadmill running activity at 60% or 80% of $\dot{V}O_2$ max did not impair DPCP induction responses, as evidenced by subsequent tissue oedema and erythema elicitation compared to resting controls.

It is unknown what effect the present RSA exercise performance has on CHS *in vivo* immune response relative to rest. In the current study, no resting control condition was undertaken, making it impossible to directly assess the effect of the RSA protocol on CHS immune responses relative to rest. However, indirect comparisons can be made. For example, Davison et al. (2015) and Jones et al. (2017) employed similar experimental designs that excluded resting controls but instead referenced third-party resting data for comparison (Diment et al., 2015). These studies concluded that a 120-minute exercise duration at 60% of $\dot{V}O_2$ max was required to impair CHS tissue oedema (inflammation) responses, with erythema remaining largely unchanged (Davison et al., 2015; Jones et al., 2017). Following this third-party

comparison approach, the summed tissue oedema responses in the present study (~3.0 mm skinfold) were compared to Diment et al. (2015) resting control data (~4.5 mm skinfold). This suggests that repeated sprint exercise induces a slightly lower tissue oedema response than seen in resting controls and aligns more closely with Diment et al. (2015) data reported for 30-minute moderate- and high-intensity exercise data (~4.2 mm and ~4.0 mm skinfold, respectively). Although speculative, these findings indicate that the repeated sprint model used in this study may have limited or no significant impact on in vivo CHS immune responses compared to resting controls. Further experimental research is needed to substantiate this conclusion with appropriate controls.

These observations when considered together with previous animal stress models (Adjei et al., 1996; Yamauchi et al., 1996; Yamauchi et al., 2002) indicate that the ‘total stress load’ imposed plays a critical role in the magnitude of CHS/DTH immunological response. In acute and chronic animal stress models, the magnitude of innate and adaptive immune system's response differs significantly to that of humans seen during acute exercise (Van der Whop, 2010; Seok et al., 2013), likely due to differences in metabolic rate (Perlman, 2016). Chronic animal stress models often induce a prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis, Chronic stress models in animals often lead to prolonged activation of the hypothalamic-pituitary-adrenal (HPA) axis, triggering distinct gene and cellular stress responses (Perlman, 2016). These chronic responses may suppress immune function and exacerbate inflammation, with potential impacts on delayed-type hypersensitivity reactions (Kulkarni et al., 2002). In contrast, acute exercise in humans induces transient increases in stress hormones, typically followed by a rapid return to baseline levels (Hill et al., 2008; Peake et al., 2017). Such differences suggest that acute human exercise-induced stress modulates immune function in a more subtle way, potentially influencing DTH responses differently, particularly when assessed using in vivo CHS methods (Long et al., 2012; Moynihan and Cohen, 2018; Bohn-Goldbaum et al., 2022). In the present study, the in vivo CHS model was unable to differentiate between placebo and intervention groups in an acute RSA exercise stress context. Consequently, this likely rendered the RSA model less ineffective in evaluating the role of nutritional interventions in mitigating exercise-related immune disruptions and prevents an assessment of any adaptogenic effects that such interventions might have.

This limitation of the present in vivo CHS model contrasts with numerous nutritional intervention studies [such as those investigating carbohydrates, glutamine, and bovine

colostrum] that have demonstrated positive effects on immune function albeit using in vitro assays rather than in vivo approaches. (Benson et al., 2012; Głowska et al., 2020; Von Ah Morano et al., 2020; Davison et al., 2021; Rosa-Neto et al., 2022). Several studies have considered the effect of nucleotide supplementation upon immune function biomarkers through the use in vitro assays. Mc Naughton et al. (2006), and Mc Naughton et al. (2007) indicated significant improvements in mucosal immunity, as indicated by increased secretory IgA (sIgA) levels, following 60 days of nucleotide supplementation and subsequent endurance (90 minutes) or short-term high-intensity (2 minutes) exercise. Similarly, Ostojic et al. (2012) reported a slowing in the post-exercise decline of sIgA levels following 14 days of sublingual nucleotide supplementation compared to placebo. However, while these studies suggest beneficial effects of nucleotide supplementation on immune function, several factors complicate attribution of these effects solely to nucleotides. These include variations in delivery methods, nucleotide content, and the presence of other immunostimulatory compounds in supplements (McNaughton et al., 2006; Ostojic et al., 2012; Sterczala et al., 2016). For instance, Cui et al. (2022) demonstrated that supplement components such as L-glutamine and inositol could serve as alternative substrates for immune cell growth and function. In addition, whilst low or absent sIgA level have historically been associated with upper respiratory tract infections they are highly variable and influenced by factors such as changes in saliva secretion rates, which were not accounted for in many of the nucleotide studies. Souza et al. (2021) observed that interval exercise-induced changes in sIgA were primarily due to compromised secretory rates rather than absolute sIgA concentrations. Additionally, sIgA levels are susceptible to fluctuations caused by non-exercise factors, including oral hygiene practices and day-to-day variations in mouth-rinsing habits (Simpson et al., 2020; Hoek et al., 2023; Okomato et al., 2023). Reflecting these complexities, Riera et al. (2013) found no significant changes in sIgA levels after a submaximal cycling exercise to exhaustion protocol following 30 days of nucleotide supplementation.

Nucleotide mediated changes in basic in vitro immune cell counts (lymphocytes, neutrophils, basophils etc) and their functional responses following exercise have also been explored. These enumeration and functional responses provide insight as to how key innate and adaptive cellular elements involved during in vivo CHS induction and elicitation may progress. For instance, mitogen stimulated lymphocyte proliferation capacity and natural killer cell cytotoxic activity have been reported to improve after acute exercise to exhaustion in populations supplemented with nucleotides (Ostojic et al., 2012; Riera et al., 2013; Casajus et al., 2014).

Casajus et al. (2014) observed enhanced lymphocyte proliferation and increased circulating CD4⁺ and CD8⁺ T-cell counts 24 hours after a 60-minute cycling session at 60-80% of $\dot{V}O_2$ max following 32 days of nucleotide supplementation compared to placebo. Riera et al. (2013) reported similar findings, with increased lymphocyte proliferation and cell counts post-exercise after 30 days of supplementation. Ostojic et al. (2012) further documented enhanced natural killer cell enumeration and activity after 14 days of sublingual nucleotide supplementation. These findings correspond with data from nutritionally compromised neonates, which have demonstrated improved natural killer cell activity following nucleotide supplementation (Carver et al., 1991). They are also consistent with substantial evidence from animal models, including porcine, avian, bovine, and aquaculture systems, showing that exogenous nucleotide supplementation enhances innate and adaptive immunity, increases disease resistance, and reduces mortality. These benefits are likely attributed to improved intracellular and extracellular signalling and the provision of nucleotides to meet the RNA and DNA synthesis demands of rapidly proliferating immune cells (van Buren et al., 1994; Cameron et al., 2001; Gill et al., 2002; Choudhury et al., 2005; Fontana et al., 2017; Reda et al., 2018; Wu et al., 2018; Dinardo et al., 2022; Gopi et al., 2023).

The evidence suggests a clear distinction in the efficacy of nucleotides when assessed using in vivo CHS methods in humans compared to in vitro immune assessments. Explanation may lie with nature, sensitivity, and specificity of these assessment approaches to detect immunological change (Albers et al., 2005; Albers et al., 2013). Challenge-based tests, such as the CHS (DPCP) test and vaccine-specific inoculations, are sensitive measures of in vivo immune response. These tests are biologically relevant and clinically significant as they capture the tissue-specific microenvironment, integrated physiological and immune interactions (Bruunsgaard et al., 1997; Vedhara et al., 1999; Albers et al., 2005; Levis et al., 2006; Frew et al., 2021). However, despite their sensitivity, in vivo tests [particularly the DPCP test used in the present study] lack the specificity needed to identify the precise immunological site or mechanism responsible for changes in immune pathways (Vedhara et al., 1999; Boraschi et al., 2021).

In contrast, in vitro immune assessments offer greater specificity. By isolating and manipulating immune cells, in vitro methods allow precise measurement of specific immune parameters, enhancing the detection of subtle changes at various stages of immune pathways (Boraschi et al., 2021). However, this specificity comes with limitations. In vitro assays, performed in isolation, fail to fully replicate the physiological complexity of in vivo immune

responses, such as those involved in CHS/DPCP tests, which limits their relevance to real-life contexts. Additionally, laboratory processes such as phlebotomy, cell separation, culturing, and harvesting introduce artifacts, including mechanical stress, cell activation, and altered proliferative kinetics. These artifacts can distort innate and adaptive immune cell enumeration and functional responses, making them inconsistent with in vivo responses and limiting the broader interpretability of in vitro data (Posevitz-Fejfar, 2014; Cossarizza et al., 2019). These limitations are evident when comparing the present study's lack of efficacy in CHS response to the positive findings noted from in vitro studies where nucleotide are present (Gill et al., 2002; Casajus et al., 2014; Ostojic et al., 2012; Riera et al., 2013). Such disparities are not unique; similar discrepancies have been observed in studies examining the efficacy of bovine colostrum and carbohydrate supplementation using in vivo and in vitro immune function assessments (Davison et al., 2015; Jones et al., 2017; Głowska et al., 2019; Davison et al., 2021). Relying solely on in vitro data without considering in vivo responses [particularly when immune function is disrupted by exercise-induced stress] may misrepresent the impact of nucleotides on human immune function particularly since nucleotide can be synthesised and salvaged in vivo to meet demand. Data from toxicology and cancer trials consistently indicate that in vitro immune responses do not always correlate with in vivo clinical endpoints (Romero et al., 2004; Bachmann and Kündig, 1994; Boraschi et al., 2021). This contrasts with the clinical relevance of DPCP assessments, which have demonstrated utility in chronic disease scenarios (Levis et al., 2006; Albers et al., 2013). To address these complexities, future research should integrate in vivo and in vitro methods to provide a more comprehensive understanding of immune responses to dietary nucleotide supplementation.

Additional mechanisms may further explain the lack of detectable effects of nucleotide supplementation on in vivo immune responses in the present study. The dose and formulation of nucleotides consumed and absorbed are consistently identified as critical factors influencing their biological effects on the immune system (Hess and Greenberg, 2012; Ding et al., 2021). The dietary ratio of nucleotide bases significantly impacts immune function, as each base has distinct roles in cellular processes and immune signalling pathways (Hossain et al., 2020; Ding et al., 2021). For instance, Yamamoto et al. (1994) demonstrated that mice fed a nucleotide-containing diet exhibited a 75% survival rate following methicillin-resistant *Staphylococcus aureus* (MRSA) infection, compared to a 33% survival rate in the placebo group. Similarly, Adjei et al. (1999) reported enhanced in vivo immune responsiveness and increased pathogen

resistance in rodents infected with *Cryptosporidium parvum* following mixed ratio nucleotide feeding compared to those on a standard diet.

These findings highlight the importance of nucleotide composition in modulating immune function. In the present study, the nucleotide formulation predominantly consisted of pyrimidines rather than purines. This balance favouring pyrimidines has been shown in rodent and aquaculture models to support immune function and disease resistance under chronic stress conditions, suggesting potential benefits of its inclusion in formulations (Kulkarni et al., 1992; Carver, 1994; Lin et al., 2009; Song and Lee, 2013; Hossain et al., 2020; Novraidi et al., 2021). For instance, Hales et al. (2002) reported that the pyrimidine uracil enhanced lymphocyte proliferation and cytokine responses in chronically stressed mice. Similarly, Yamauchi et al. (1996) and Yamauchi et al. (2002), using in vivo delayed-type hypersensitivity immune assessments, demonstrated improved responses when animals were provided with nucleotide supplements, either as individual mononucleotides or as a mixture. Kulkarni et al. (2002) further supported the role of pyrimidine supplementation in enhancing cell-mediated immune responses during simulated microgravity stress. Based on these findings, the pyrimidine-based formulation used in this study was expected to influence immunity; however, it did not as no detectable alterations in CHS immune response outcomes were observed.

Consequently, the current chapter data cannot confirm whether this formulation is effective in maintaining immunity in humans subjected to short-term acute RSA exercise stress. A fundamental limitation lies in the translation of results from animal models to humans. In many animal studies, the effectiveness of nucleotides is based on comparison to preformulated diets (controls) in which nucleotides are not a primary constituent or have been experimentally restricted or entirely removed. Such restrictive dietary manipulations are known to impair a wide range of innate and adaptive immune responses in animals (Carver et al., 1994; Yamauchi et al., 1996; Jyonouchi et al., 1996; Adjei et al., 1999; Xu et al., 2013; Tie et al., 2021; Scherer et al., 2023). Such approaches act to maximise the potential difference(s) between intervention and control, which may be ecologically invalid. This approach does not reflect human dietary patterns, as humans cannot be completely nucleotide restricted and can obtain nucleotides from a variety of animal and plant sources acting to provide an exogenous contribution to the endogenous de novo and salvage pathways. Further, since nucleotide content in foods is not typically quantified, unquantified amounts of purine and pyrimidine nucleotides may be introduced into the experimental design. The potential impact of this upon experimental

outcomes i.e., in vivo immunity remains uncertain (van Buren et al., 1994; Sauer et al., 2011; Hess and Greenberg, 2012; Ding et al., 2021).

Furthermore, differences in experimental stress model implemented are a critical factor. It is plausible that the stress induced by RSA exercise was insufficient to exceed the individual's endogenous pathways capacity to provide nucleotides. In high stress animal models [characterised by continual physiological and psychological challenges] this may amplify the differences between nucleotide-supplemented and nucleotide-restricted or nucleotide-free diets. Together, these factors of dietary restriction and sustained elevated stress levels artificially maximise the distance between the imposed functional nucleotide demand and the capacity of endogenous *de novo* and salvage pathways to meet it, thereby accentuating the importance of dietary nucleotide supplementation in these models (Xu et al., 2017; Ding et al., 2021). In this study, it is unlikely that such extreme stress conditions were replicated. It is therefore plausible that only under high training load models, involving sustained physiological and psychological stress, impaired sleep and recovery, and poor nutrition, would dietary nucleotide supplementation demonstrate clear efficacy. Future research employing such models is needed to better elucidate the potential benefits of nucleotide supplementation under more extreme and prolonged stress conditions. Comparisons of nucleotide efficacy between human and animal models may be somewhat problematic based on this gulf in stress conditions used to demonstrate effectiveness.

Similarly, other biomarkers of stress may support such an argument. Acute high-intensity repeated sprint exercise (RSA), as implemented in the present study, led to a transient activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in elevated cortisol concentrations during and after exercise across the participants in this work. The cortisol response observed aligns with the magnitude and temporal pattern documented in previous studies on high-intensity interval training (HIIT) or RSA protocols. Specifically, cortisol levels increased significantly between 0–60 minutes post-exercise, began declining toward baseline between 120–180 minutes, and returned to baseline within 24 hours (Hill et al., 2008; Dote-Montero et al., 2021). Importantly, there were no significant differences in cortisol levels between the nucleotide and placebo conditions at any time point, either before or after RSA activity. This finding is consistent with the exercise performance and physiological load data, which indicate comparable stress levels between the two conditions.

These results contrast with prior studies reporting attenuated HPA axis activation following nucleotide supplementation in exercise contexts. For example, Mc Naughton et al. (2006) and Mc Naughton et al. (2007) demonstrated that dietary nucleotide supplementation (450 mg·day⁻¹ for 60 days) reduced cortisol concentrations after 90 minutes of cycling and 2 minutes of high intensity cycling, respectively. Similarly, Sterczala et al. (2016) found that 14 days of nucleotide supplementation mitigated cortisol increases following acute heavy resistance exercise. Although the present study employed a shorter supplementation period (30 days) but a higher daily dose (~2500 mg·day⁻¹), this present nucleotide dosing protocol did not suppress cortisol levels before or after RSA activity. Notably, the nucleotide supplements used in these previous studies contained additional bioactive components, which may have contributed to their effects on HPA axis activation. This raises the possibility that the observed attenuation of cortisol in these studies cannot be attributed solely to nucleotides but may involve synergistic effects from other bioactive ingredients.

The observed differences in cortisol responses between this and other nucleotide supplementation studies may also be attributed to variations in the modality, intensity, and duration of exercise protocols relative to those employed in the present investigation. For example, Dote-Montero et al. (2021) highlighted significant variations in cortisol responses following high-intensity interval training (HIIT) protocols with differing work-to-rest ratios and effort durations. In animal models, particularly porcine and aquatic species, nucleotide supplementation has demonstrated the ability to attenuate HPA axis activation (reflected in reduced cortisol increases) in response to various chronic environmental, housing, handling, and catch-release stressors (Yamauchi et al., 2002; Palermo et al., 2013; Tahmasebi-Kohyani et al., 2012; Superchi et al., 2012; Jiao et al., 2018; Hossain et al., 2020; de Cruz et al., 2020; Upadhaya and Kim, 2020; Gatlin et al., 2022).

The mechanism(s) by which dietary nucleotides modulate cortisol responses in humans remain poorly understood. However, during exercise, where energy requirements are elevated the attenuation in cortisol release noted by Mc Naughton et al. (2006) and Mc Naughton et al. (2007) and Sterczala et al. (2016) could be linked to the ability of adenosine and uracil to increase hepatic glucose output and energy status (Yamauchi et al., 2002; Gella et al; 2008; Hess and Greenberg 2012; Xu et al 2017). During exercise, reductions in blood glucose stimulates cortisol secretion to increase gluconeogenesis and glycogenolysis (Hackney and Walz 2013). If nucleotide supplementation were to increase hepatic glucose output in humans,

as demonstrated in animal models (Xu et al., 2017), it could theoretically maintain or elevate blood glucose levels, thereby reducing the physiological need for cortisol elevation. Additionally, it has been suggested that dietary nucleotides may upregulate neurotransmitter synthesis in the hypothalamus, potentially altering the stress response by downregulating the release of corticotropin-releasing factor (CRF) and pro-opiomelanocortin (POMC), as evidenced in fish models (Welker et al., 2011; Palermo et al., 2013). However, no corresponding data are available in humans. The current findings do not support the hypothesis that dietary nucleotide supplementation attenuates cortisol release in response to acute repeated sprint activity, as no differences were observed between the intervention and placebo conditions. Given the broad responses noted across individual participants, further research is necessary to elucidate the potential mechanisms and contexts under which nucleotides may modulate cortisol responses in humans.

In the context of cortisol's impact on immunity, it plays a pivotal anti-inflammatory role in response to exercise by suppressing pro-inflammatory signalling and immune cell activation (Walsh et al., 2011; Peake et al., 2014; Moynihan & Cohen, 2018). Specifically, cortisol influences the maturation and migration of skin-derived dendritic cells to lymph nodes, impairing cytokine production and the priming of CD8⁺ T cells *in vivo*, which are critical processes in the induction of contact hypersensitivity (Elftman et al., 2007; Liberman et al., 2018). When cortisol levels are elevated, nucleotides have been shown to counteract these effects by enhancing the differentiation, proliferation, and activation of CD4⁺ and CD8⁺ T cells, as well as other immune cells. This is achieved through increased cytokine production, stimulation of lymphoproliferative responses, and improvements in disease resistance (Adjei et al., 1999; Kulkarni et al., 2002). In the present study, where no significant differences in cortisol release or CHS immune response were observed between nucleotide and placebo treatments, it appears that nucleotides did not play a substantial role in modulating CHS responses through alterations in cortisol levels. This finding aligns with prior research reporting no association between cortisol, adrenaline, or noradrenaline levels and changes in CHS induction or elicitation responses following exercise protocols lasting between 30 minutes and 2 hours (Diment et al., 2015; Davison et al., 2015).

The interpretation of the effects of nucleotide supplementation on cortisol levels and CHS immune responses is influenced by the sampling regimen employed. In this study, cortisol samples were collected pre- and post-exercise, as well as up to 24-hours after exercise.

However, the time frame for CHS induction responses can span several days to weeks, as it requires progression through multiple biological steps to establish immunological memory (Field, 1996; Friedman, 2006). Consequently, no stress hormone data were collected beyond this immediate post-exercise period. Any physical or psychological stressors occurring during this unmonitored window could potentially influence the strength of individual CHS induction and the consolidation of immune memory (Edwards et al., 2018). Moreover, cortisol levels were not measured during the DPCP elicitation phase, which serves to measure the strength of CHS induction. Elevated stress hormones during this phase could modulate the individual's immune response elicited by subsequent DPCP exposure. Given the ~28-day interval between the induction and elicitation phases, which remained unmonitored, the potential for confounding factors affecting the individual's elicitation outcomes increases. This limitation in experimental design is not unique to the current study but is also present in the majority of in vivo human CHS exercise studies (Harper-Smith et al., 2011; Oliver et al., 2013; Diment et al., 2015; Davison et al., 2015; Jones et al., 2015). Future research should aim to measure stress hormones during both the induction and elicitation phases to address this limitation.

Several additional limitations in the current study warrant consideration, including those related to experimental design, in vivo CHS measurement of oedema and erythema, dietary factors, and psychological stress. The cross-sectional design used in this study, though logistically practical for data collection, introduces inter-participant variability that may obscure subtle individual effects of nutrition on immune responses and thereby impact the assessment of individual nucleotide efficacy. Specifically, treatment allocations were not based on the primary outcome variable (CHS) response due to the constraints of the DPCP in vivo model, which limits its application to a single induction and elicitation exposure. This cross-sectional approach has been widely adopted in CHS in vivo studies examining the effects of exercise and nutritional interventions on immune responses (Diment et al., 2015; Davison et al., 2015; Jones et al., 2017). However, it differs from the repeated-measures design used by Harper-Smith et al. (2011), which requires several months of repeated exposure to consolidate immunological responses (Harper-Smith et al., 2011; Mose et al., 2017). The cross-sectional approach also increases the likelihood of free-living artifacts [factors outside the control of the study] that could alter immunological competence to DPCP exposure in each individual participants lived experience. For example, clinical or sub-clinical bacterial or viral infections may have priming or inhibitory effects on immune response depending on the type and timing

of exposure (Biram et al., 2022). These considerations highlight the importance of controlling external variables and refining experimental designs in future nucleotide research.

The present study also highlights a key limitation associated with the use of morphological markers as surrogates for underlying immunological processes, raising questions regarding their validity and reliability as indices of immunity. Several factors may influence these assessments. For instance, the erythema (skin redness) index, as measured by colourimetry, is subject to limitations stemming from variations in skin colour and pigmentation (Partl et al., 2017). Moreover, the use of erythema and oedema measurements may be affected by immune-mediated responses such as vesicle/blister formation at exposure sites where strong reactions occur. Such responses, particularly yellow vesicles and fluid-filled blisters, can impair the accuracy of erythema and thickness readings (Figure 4.1.7). Vesicle and blister formation is commonly associated with strong CHS reactions to DPCP, driven by high levels of CD4⁺ helper and cytotoxic CD8⁺ T-cell infiltration in the epidermis (Pinchler, 2003; Wick, 2017; Copaescu, et al., 2021). These limitations are well-documented in the literature regarding CHS methodologies (Harper-Smith et al., 2011; Davison et al., 2015; Diment et al 2015; Jones et al., 2017). Current findings show vesicle occurrence predominated in the higher dose response curve range across all participants indicating the presence of a strong induction response; the magnitude of vesicle size and colouration varied widely across each individual participants with clear responder non-responder phenotype expression.

In the current study, vesicle occurrence was observed in individual participants exhibiting higher responses along the dose-response curve, indicating a strong induction response. However, the size and colouration of vesicles varied substantially between individuals across the intervention groups. Consistent with recommendations from Harper-Smith et al. (2011) and Davison et al. (2015), it is suggested that oedema data should serve as the primary indicator of CHS responses, with erythema treated as a secondary outcome measure. While other researchers disagree with relegating erythema to a secondary role, they caution that its interpretation may be unreliable at sites where vesicle formation occurs (Diment et al., 2015; Frew et al., 2021).

Additionally, anatomical variation in CHS responses warrants consideration. Schmidt et al. (2016) demonstrated that the site of induction and subsequent re-exposure to contact sensitizers significantly influences both erythema and oedema responses. Their research, conducted in

both mouse and human models, revealed that presenting induction and elicitation challenges at the same anatomical site or region elicited a stronger site-specific and systemic immunological memory response. Conversely, when induction and elicitation occurred at distinct anatomical regions, the amplitude of the systemic immunological memory response was significantly diminished. In the context of the present study, these findings suggest that a stronger elicitation response may have been achieved by re-challenging the sensitised lower back region rather than using the anatomically distinct medial aspect of the upper arm. This consideration should inform the design of future trials. However, it is important to note that even with such variations in response strength, between anatomical locations the observed effects remain clinically relevant in disease models (Albers et al., 2005; Levis et al., 2006). Importantly, regardless of whether erythema or oedema data are used, the overarching conclusion of the study remains unchanged. Specifically, repeated sprint activity (RSA) exercise, following 30 days of nucleotide ingestion, does not appear to alter CHS-determined *in vivo* immunity.

The free-living nature of the experimental design precluded the standardisation of participant's diets throughout the study. Participants were instructed to record their food intake during the 24 hours preceding the exercise on the DPCP induction (sensitisation) day and replicate this dietary pattern on the DPCP elicitation day. However, the nucleotide intake from individual participants habitual diets was not quantified, as commonly used nutritional analysis software does not report purine and pyrimidine content. Consequently, in a heterogeneous population such as that in the current study, basal purine and pyrimidine intake levels from their diet may have varied significantly between individuals. As such this may predispose to a responder non-responder phenotype that was not adequately captured in the minimisation of difference allocation protocols.

Although assessing nucleotide pool levels in red blood cells via blood samples could have provided a marker of bioavailability, this was not feasible in the present study, despite such samples having been collected, processed for analysis. Furthermore, the participants in this study exercised in a fasted state prior to DPCP induction, consistent with the methodology adopted by Jones et al. (2017). In contrast, other studies have utilised non-fasted protocols (Oliver et al., 2013; Davison et al., 2015; Diment et al., 2015). Davison et al. (2015) suggested that fasting may impair immune responses *in vitro* by increasing cortisol and stress hormone secretion due to limited carbohydrate availability, although this finding has not been universally supported (Allgrove et al., 2007).

While the specific effects of an overnight fast on CHS immune responses have not been comprehensively investigated, the combined effects of fasting and exercise are known to influence various aspects of immune function and inflammatory responses in vitro (Hance et al., 2007; Mooren et al., 2020; Wilhelm et al., 2021; Nobari et al., 2022). Reductions in circulating glucose and glutamine [both of which are critical for antigen-presenting cell function, T-cell activation, and effector function] could potentially alter the magnitude and duration of CHS induction and the consolidation of immune memory. Low glutamine also impairs *de novo* nucleotide synthesis. Additionally, elevated secretion of pro-inflammatory cytokines such as TNF- α and IL-6 has been observed in fasted states, which may further impact CHS induction and elicitation. Mooren et al. (2020) found that overnight fasting prior to 30–60 minutes of aerobic exercise at 60% of $\dot{V}O_{2\max}$ increases T-cell apoptosis and impairs cell proliferation. Given the importance of T cells in the CHS challenge test, such effects could potentially weaken the strength of the individual CHS induction response. Although no significant differences were observed between the nucleotide and placebo groups in the current study, it cannot be definitively ruled out that the fasted state adversely influenced the magnitude of each individual's CHS induction response. Nonetheless, any potential disruptions caused by fasting were likely to have affected both cohorts similarly, however the distribution of participants, habituated and/or exposed to prior fasted exercise activity was unquantified and should be acknowledged as a confounder.

An important consideration in this study pertains to the phase and duration of when the nutritional supplementation occurred. Unlike Jones et al. (2017), who supplemented participants with bovine colostrum both prior to the induction phase and through the elicitation phase (~58 days), the current study provided nucleotides or placebo only up to the start of the induction phase, discontinuing supplementation thereafter. This approach was informed by evidence suggesting that a strong induction response is essential for robust CHS elicitation responses. Increasing nucleotide availability during the pre-induction window was intended to optimise potential adaptogenic effects before initial DPCP exposure (Poenaru and Christou, 1991; Field, 1996; Friedman, 2007). However, the present study found no significant differences in CHS responses between the nucleotide and placebo groups. Some insights may be drawn from Jones et al. (2017), who also found no effect of bovine colostrum supplementation on overall CHS responses, suggesting that continuation of supplementation across both the induction and elicitation phases might have minimal impact. Since bovine colostrum is naturally rich in nucleotides (Playford and Weiser, 2021), this may indirectly

reflect the potential efficacy of prolonged nucleotide supplementation. However, uncertainty persists, as to whether extending nucleotide supplementation through to the elicitation phase might have yielded a differentiation in CHS responses between the nucleotide and placebo groups. Future research should explore whether extending nucleotide supplementation up to the elicitation phase could further support CHS responses.

Another limitation of this study is the lack of quantification of individual participants' psychological stress levels during the CHS induction and elicitation phases. Psychological stress, whether acute (state anxiety) or chronic (trait anxiety), can significantly influence both *in vitro* and *in vivo* immune function, altering immune regulation, surveillance, and disease expression in both positive and negative ways (Jemmott et al., 1983; Dantzer and Kelley, 1989; Khansari et al., 1990; Deinzer et al., 2000; Morey et al., 2015; Drew et al., 2017; Hayward et al., 2020). For example, exam stress has frequently been used as a 'challenge' intervention capable of modifying immune function (Jemmott et al., 1983; Murphy et al., 2010; Kulsoom et al., 2015; Turner et al., 2020). This is particularly relevant to the present study, as all participants were undergraduate or postgraduate students assessed during their academic terms, which likely exposed them to varying degrees of individual academic and examination stress. Such stress could have influenced their individual immune responses during the induction and elicitation phases (Bosch et al., 2004; Kulsoom et al., 2015; Turner et al., 2020). Vaccine challenge studies similarly demonstrate a negative association between heightened anxiety and *in vivo* antibody responses to the vaccination (Dhabhar, 2008; Levi et al., 2011; Hayward et al., 2020). Moreover, academic stress has been shown to impact immune function differently within diverse student cohorts, a phenomenon likely applicable to the participants in this chapter who were at different stages of their academic degree i.e., first year vs final year students (Al-Dabal et al., 2010).

These effects may be mediated by stress hormones such as cortisol and catecholamines, which can modulate both innate and adaptive immunity (Carrasco and Van de Kar, 2003; Moynihan and Cohen, 2018). While no differences in resting or exercise-induced cortisol responses were observed between the nucleotide and placebo groups, findings from Edwards et al. (2018) highlight the critical influence of pre-exercise psychological stress on CHS immune responses. Edwards et al. (2018) demonstrated that pre-exercise state anxiety levels explained differences between *in vivo* immune responses to exercise-induced stress (2-hour exercise stress @ 60% of $\dot{V}O_2$ max), as strongly as the correlation between physiological stress markers and CHS in

vivo immunity. These findings suggest that pre-exercise psychological stress plays a pivotal role in modulating immune responses to exercise. Consequently, future studies utilising CHS to assess in vivo immunity should consider the influence of the individual's psychological stress at induction, elicitation, and throughout any nutritional supplementation period. Importantly, previous research examining CHS-mediated immune responses in the context of nutritional or exercise interventions has not accounted for this variable (Harper-Smith et al., 2011; Diment et al., 2015; Davison et al., 2015; Jones et al., 2017).

4.6 Conclusions

This study represents the first attempt to evaluate the effects of chronic dietary nucleotide supplementation on in vivo immune responses following repeated sprint activity (RSA) exercise. The findings indicate no significant changes in nucleotide-related immunological or hormonal responses to RSA exercise relative to placebo. This suggests the immune system was not sufficiently challenged under this study's conditions to elicit/express any potential nucleotide-mediated enhancements. While previous research in animal models has demonstrated improvements in immune function, and disease resistance through nucleotide supplementation, such effects were not observed in this human study under the specific dosage and formulation employed. The results further imply that the RSA exercise model used may not have generated enough physiological stress to disrupt immune homeostasis. Consequently, the immune system maintained effective surveillance, activation, and memory functions, reducing the likelihood of detectable benefits from exogenous nucleotide supplementation. In this context, introducing additional nucleotides as an adaptogenic agent appears unlikely to influence immunological function, as the immune system was operating efficiently. Future research should carefully consider the exercise-induced physiological loading necessary to challenge or exceed the immune system's homeostatic balance. Incorporating more homogenous within-subject population designs, especially in studies employing in vivo contact hypersensitivity (CHS) challenges, will be essential, but logistically challenging. Additionally, verification of nucleotide effect through in vitro assessments of cellular enumeration and functional response changes should be included. These methodological refinements are critical for determining whether dietary nucleotide supplementation can enhance immune function in exercising humans.

Chapter 5

Effect of Dietary Nucleotide Supplementation on in vitro C₂ C₁₂ Skeletal Muscle Cell Growth. (Study 4)

Chapter 3 and 4 of this thesis presented the first assessment of chronic dietary nucleotide supplementation on gastrointestinal functional biomarkers and in vivo immune responses following repeated sprint activity (RSA) exercise. These data indicate no significant changes in nucleotide-related gastrointestinal, immunological, or hormonal responses, suggesting that the exercise stimulus may not have sufficiently disrupted homeostatic balance in these systems to allow for the adaptogenic effects of nucleotides to be expressed. It is plausible that the physiological stress induced by the RSA protocol was too mild to elicit the kind of physiological challenges where nucleotide supplementation might confer benefit. To address this limitation, the following chapter transitions to an in vitro model using murine C₂C₁₂ muscle myoblast cells subjected to an externally generated inflammatory stress model. Unlike the in vivo RSA model, this in vitro approach allows for precise induction and control of a robust stress environment in proliferating and differentiating tissue. This creates a more sensitive platform to evaluate the potential protective and regenerative effects of nucleotides under defined catabolic conditions, as mediated by TNF- α ; a cytokine when present at high concentrations is known to suppress myogenesis, promote muscle atrophy, and disrupt cellular growth processes.

5.1 Abstract

Exogenous nucleotides (NTs) are considered conditionally essential nutrients, under conditions of high stress. Therefore, the external supplementation of exogenous nucleotides is of significance for maintaining normal cell proliferation and growth. This study, therefore, sets out to assess the effect of a commercial mixture of the nucleotides upon morphological changes in C₂C₁₂ myoblast and to elucidate the potential mechanism through understanding changes in mRNA signalling. A C₂C₁₂ mouse skeletal myoblasts cell line was treated with different exogenous nucleotides at two doses 31.25 µg·mL⁻¹ and 62.5 µg·mL⁻¹ with DMSO at 0.02 and 0.04%, respectively. Dose were previously determined from a dose response toxicity analysis. Cell treatments continued for up to 72 hours for gene expression analysis and 7 days for morphological analysis. Morphological analysis included assessment of myotube number, myotube diameter and area, and gene expression analysis of genes associated with myogenesis (*myoD*, *myogenin*), growth factors known to regulate differentiation (*Igf-1*, *Igfbp2*, *Igfbp5*) and transcripts associated with TNF-α signalling, apoptosis and muscle atrophy (*TNF-α*, *Tnfrsf1a*, *Tnfrsf11b*, *Murf-1*, *Mafbx-1*, *Myostatin*, *Fox01*, *Fox03*, *NF-κb*, *p53*). To assess myotube atrophy wells were co incubated with TNF-α at 20 ng·mL⁻¹ under nucleotide treated and untreated conditions. Data indicates that morphological parameters myotube numbers were not significantly different between nucleotide incubation doses ($F_{3,16} = 4.10$, $P = 0.06$). However, myotubes numbers ($F_{3,16} = 6.54$, $P = 0.04$), diameters ($F_{3,16} = 3.54$, $P = 0.04$) and areas differed by treatment condition. Generally, a step wise reduction in myotube number, diameter and area was observed with an adverse synergistic effect apparent when TNF-α and nucleotide were co incubated particularly at the higher nucleotide doses. Gene expression differed widely across all mRNA with no clear pattern of gene upregulation of change across the three areas relating to myogenesis differentiation and TNF-α signalling, apoptosis and muscle atrophy. In the current C₂C₁₂ mouse skeletal myoblasts model nucleotides failed to maintain cell growth and proliferation or alter gene expression in a coherent manner consistent with improvements in these parameters when subject to inflammation stress. It is apparent that TNF-α co-incubation with a mixed purine and pyrimidine formulation lead to impairments in both myotube number, diameter, and area. Further work is required to determine whether individual nucleotide may act differently and quantify the optimal incubation doses for each in skeletal muscle.

5.2 Introduction

Nucleotides are essential biomolecules required for the synthesis of DNA and RNA, playing a crucial role in cellular proliferation and growth (Lane and Fan, 2015; Ding et al., 2021; Diehl et al., 2022). As non-protein nitrogen-containing compounds, nucleotides support growth in both humans and animals. As the fundamental building blocks of nucleic acids, nucleotides are indispensable for genetic material replication during cell division and transcription processes essential for protein synthesis (Rudolph, 2005; Ferrier, 2014). Proliferating cells exhibit a high demand for nucleotides to support RNA synthesis and ensure precise DNA replication during the S phase of the cell cycle. Without sufficient nucleotide availability, cells are unable to replicate or repair DNA effectively, compromising metabolic functions that sustain proliferation and growth. During DNA synthesis, nucleotides [specifically deoxyribonucleotides (dATP, dGTP, dCTP, and dTTP)] form the structural framework of the DNA double helix. Similarly, ribonucleotides (ATP, GTP, CTP, and UTP) are critical for ribosomal RNA and messenger RNA synthesis, facilitating the translation of genetic information into functional proteins that enable biomass production (Hess and Greenberg, 2012; Dhiel et al., 2021).

Traditionally, nucleotides were not classified as essential nutrients because they can be synthesised *de novo* by most tissues under normal physiological conditions (Hess and Greenberg, 2012; Ding et al., 2021). However, certain cells lack adequate endogenous synthesis capacity and rely on exogenous nucleotide sources to mitigate deficits (Van Buren et al., 1994; Gil et al., 2002; Zhu et al., 2024). For instance, in conditions of skeletal muscle stress, endogenous nucleotide production may become insufficient (Thompson et al., 1978; Yoshihara et al., 2006; Miller et al., 2019; Uberti et al., 2020). Nucleotide supplementation has proven particularly beneficial during periods of rapid growth, stress, or tissue repair, where nucleotide scarcity impairs cell growth as a protective mechanism to conserve intracellular nucleotide resources (Diehl et al., 2012). In cases where endogenous synthesis fails to meet heightened demand, exogenous nucleotide supplementation becomes critical. Evidence demonstrates that such supplementation enhances cellular proliferation and growth across diverse systems, including stem cells (Carver et al., 2004), enterocytes (Sauer et al., 2012; Tie et al., 2021), hepatocytes (Perez et al., 2004), lymphocytes (Salobir et al., 2005), and skeletal muscle (Uberti et al., 2020; Wang et al., 2024).

Animal studies corroborate the role of nucleotide supplementation in promoting tissue repair, growth, and muscle protein deposition, thereby improving carcass yield. Research in avian (Wang et al., 2021), bovine (Dehghan-Banadaky et al., 2013), porcine (Davila-Ramirez et al., 2020), and aquaculture models (Andriamita et al., 2020) highlights these benefits (Yin et al., 2015; Xu et al., 2013; Rahmawati et al., 2021; Ciurescu et al., 2021). In malnourished human infants, nucleotide supplementation similarly supports growth during critical developmental stages (Cosgrove et al., 1996; Singhal et al., 2010; Wang et al., 2019). Conversely, when purine nucleotide availability is restricted in skeletal muscle tissue either in vivo or in vitro, muscle atrophy is observed (Miller et al., 2019; Powrózek et al., 2022). Conversely, augmenting intracellular purine levels [either through enhanced salvage or inhibition of breakdown pathways] improves muscle quality and strength (Thompson et al., 1978). In aquaculture, Wang et al. (2024) demonstrated that dietary nucleotide supplementation increased muscle fiber number and improved growth and muscle quality in fish. Mechanistic insights suggest that nucleotides exert their effects through antioxidant and anti-inflammatory properties, promoting cellular growth, repair, and differentiation across physiological systems (Salobir et al., 2005; Hossain et al., 2016; Xu et al., 2013, 2017; Ti et al., 2019; Tie et al., 2021; Zhu et al., 2021). For instance, nucleotides can attenuate pro-inflammatory cytokine expression while enhancing anti-inflammatory cytokine levels in skeletal muscle, the gut, and immune tissues. These findings underscore their potential as therapeutic agents to counteract inflammation and oxidative stress.

These findings highlight the potential role of dietary nucleotides in enhancing physiological growth and recovery across species. For example, in vitro studies of C₂C₁₂ mouse myoblasts have demonstrated increased cell proliferation, differentiation, and growth when nucleotides are supplemented in the growth medium (Uberti et al., 2020; Wu et al., 2024). Similarly, Zhu et al. (2024) reported neuroprotective effects of exogenous nucleotides in pheochromocytoma (neuron) cell lines when nucleotides were incubated in the culture medium. Mechanistically, nucleotides are proposed to exert their effects on cell proliferation, differentiation, and growth through several mechanisms including enhanced antioxidant and anti-inflammatory pathways together with increased availability for cell replication. Numerous animal studies corroborate these findings, reporting increased antioxidant activity and elevated expression of anti-inflammatory cytokines, alongside reduced levels of pro-inflammatory cytokines in the gut, immune tissues, and skeletal muscle (Salobir et al., 2005; Hossain et al., 2016; Xu et al., 2013; Xu et al., 2017; Ti et al., 2019; Tie et al., 2021; Zhu et al., 2021).

Following exercise, the levels of mechanical and oxidative stress rise in proportion to the intensity, duration, and modality of the exercise activity performed. This triggers a transient systemic inflammatory and oxidative stress response (McGee and Hargreaves, 2020). However, such post-exercise responses can impair performance and reduce skeletal muscle function, particularly when the exercise is novel or when there is insufficient recovery time between repeated bouts (Cheng et al., 2021). While acute inflammation is necessary for muscle adaptation, repair, and growth, sustained oxidative stress and chronic inflammation can result in skeletal muscle dysfunction, compromising muscle performance, repair, and regeneration (Dumond et al., 2015; McGee and Hargreaves, 2020). Repeated exposure to exercise-induced stress with limited time for adaptation has been shown to create an intramuscular environment of chronic inflammation, characterised by heightened immune cell activity and increased production of pro-inflammatory cytokines such as TNF- α , IFN- γ , and IL-6 (Chen et al., 2020; McGee and Hargreaves, 2020).

Tumour Necrosis Factor-Alpha (TNF- α) is recognised as a key regulator in the inflammatory cytokine cascade, often referred to as a "master regulator" of cytokine production (Parameswaran et al., 2011). In skeletal muscle tissue, TNF- α facilitates vascular permeability, immune cell infiltration, and the clearance of necrotic tissue following injury or damaging exercise. At low concentrations, TNF- α promotes cell proliferation through the activation of MAPK pathways, as evidenced by studies demonstrating early increases in proliferation following TNF- α exposure (Al-Shanti et al., 2008; Serrano et al., 2008; Sharples et al., 2010). Additionally, TNF- α has been positively correlated with satellite cell activation *in vivo* after damaging exercise (Mackey et al., 2007; Mikkelsen et al., 2009; Van De Vyver et al., 2012). However, chronic, or excessive TNF- α exposure is associated with adverse effects, such as increased apoptosis and impaired muscle function (Saini et al., 2008; Sharples et al., 2010; McGee and Hargreaves, 2020). This duality underscores the concentration-dependent effects of TNF- α , with low levels mediating repair and high levels exacerbating damage (Alvarez et al., 2024). Moreover, inflammation is further intensified by the production of reactive oxygen species (ROS).

Collectively, while nucleotide supplementation has demonstrated anti-inflammatory, antioxidant, and growth-promoting effects in various animal models, no studies have specifically investigated the potential of nucleotides to mitigate skeletal muscle inflammation and promote cell proliferation and differentiation under conditions mimicking chronic

exercise-induced inflammatory stress. Previous studies have shown that glutamine, a precursor in the de novo synthesis of nucleotides, can ameliorate inflammation-induced impairments in muscle cell differentiation and reduce myofiber atrophy under TNF- α stress (Girven et al., 2016). Notably, pigs fed with nucleotides exhibit similar benefits to those receiving glutamine supplementation, particularly in suppressing TNF- α production; an effect that appears consistent across species (Amorim et al., 2017; Vijayaram et al., 2023). This raises the question of whether nucleotides might exert comparable anti-inflammatory effects in an invitro TNF- α -induced stress model. The aim of this study was to investigate whether nucleotide supplementation in the presence of TNF- α could preserve C₂C₁₂ myoblast differentiation and growth, while also determining changes in regulatory gene expression associated with these processes. It is hypothesised that nucleotides will serve as a regulator of TNF- α -induced inflammatory stress, attenuating its detrimental effects on C₂C₁₂ myoblast differentiation and myotube atrophy, as observed in prior studies involving chronic TNF- α exposure (Sharples et al., 2010; Sharples et al., 2016; Girven et al., 2016).

5.3 Methods

5.3.1 Cell Culture and Treatments

C₂C₁₂ mouse skeletal myoblasts (Blau et al., 1985; Yaffe and Saxel, 1977) were used below passage 12. T₇₅ flasks were seeded with C₂C₁₂ cells at a density of 1×10^6 in growth media (GM) composed of: DMEM supplemented with: 10% hi (heat-inactivated) fetal bovine serum, 10% hi newborn calf serum, 1% L-glutamine (2 mM final), and 1% penicillin-streptomycin solution and grown to approximately 80% confluency. Six well plates were pre-coated with 1 mL of 0.2% gelatine and incubated for 15 minutes at room temperature. For cell plating, adherent cells on T₇₅ flasks were collected following trypsinisation, counted and seeded at 8×10^4 cells.mL⁻¹ in 2 mL GM per well of a six well plate and incubated for 24 hours. To assess the impact of early differentiation/fusion, C₂C₁₂ myoblasts were washed twice with PBS and dosed with 2 mL differentiation media (DM) composed of DMEM supplemented with 2% horse serum, 1 % L-glutamine (2 mM), and 1 % penicillin-streptomycin solution (DM) and 4 experimental conditions: 1) DM vehicle (DM), 2) Nucleotides (N), 3) TNF- α at 20 ng·mL⁻¹ (α), and 4) Nucleotides + TNF- α (N α), repeated for 2 doses of nucleotides 31.25 μ g·mL⁻¹ and 62.5 μ g·mL⁻¹. Because nucleotide doses at 31.25 μ g·mL⁻¹ and 62.5 μ g·mL⁻¹ contained DMSO at 0.02 and 0.04% respectively used to reconstitute the nucleotides into solution. These levels of DMSO were also contained in all of the 4 conditions described above. To further assess the

impact on established myotube cultures, C₂C₁₂ cells were cultured in DM for 7 days to induced myotube formation then dosed as above (DM, N, α , N α) and cultured for a further 72 hours for gene expression analysis and for morphological analysis (up to total of 10 days post low serum transfer). Morphological analysis included assessment of myotube number, myotube diameter and area, (described below) and gene expression analysis of genes associated with myogenesis (*myoD*, *myogenin*), growth factors known to regulate differentiation (*Igf-1*, *Igfbp2*, *Igfbp5*) and transcripts associated with TNF- α signalling, apoptosis and muscle atrophy (*TNF- α* , *Tnfrsf1a*, *Tnfrsf1b*, *Murf-1*, *Mafbx-1*, *Myostatin*, *Fox01*, *Fox03*, *Nf- κ b*, *p53*). Nucleotides were purchased from Nucleotide Nutrition, UK and TNF- α from Merck Millipore, UK. All cell experiments were repeated on 3 separate occasions. It is worth noting that an initial dose response of nucleotides was administered to differentiating cells prior to the main experimental dosing conditions to ascertain how the varying doses of DMSO (to be used later as the vehicle of delivery in nucleotide experiments) affected cell viability and differentiation/myotube formation. Doses of 125, 250 and 500 $\mu\text{g}\cdot\text{mL}^{-1}$ nucleotides containing 0.0825, 0.165 and 0.33% DMSO appeared toxic with elevated cell death distinguished by a large frequency of detached cells under the light microscope. However, lower doses of 31.25 and 62.5 $\mu\text{g}\cdot\text{mL}^{-1}$ of nucleotides (0.02 and 0.04% of DMSO respectively) did not negatively impact on differentiation and myotube size and furthermore no toxicity was detected, therefore as detailed above nucleotide doses of 31.25 and 62.5 $\mu\text{g}\cdot\text{mL}^{-1}$ were taken forward into dosing experiments with the inflammatory cytokine, TNF- α . In line with the above aim and hypothesis, this would allow us to assess if these doses of nucleotides could prevent the reductions in differentiation and myotube growth associated in the presence of inflammatory cytokine, TNF- α . It is worth noting that 20 $\text{ng}\cdot\text{mL}^{-1}$ TNF- α has been previously used extensively by ourselves and others to investigate inflammation induced cell death, reductions in muscle cell differentiation and myotube growth in C₂C₁₂ myoblasts (Foulstone et al., 2001; Stewart et al., 2004; Tolosa et al., 2005; Saini et al., 2008; Sharples et al., 2010). C₂C₁₂ cell lines demonstrate similar myotube atrophy at 20 $\text{ng}\cdot\text{mL}^{-1}$ (Sharples et al., 2010, Girven et al., 2016) to that observed in human myotubes at 10 $\text{ng}\cdot\text{mL}^{-1}$ (Foulstone et al.), with 10 $\text{ng}\cdot\text{mL}^{-1}$ occurring systemically in inflamed muscle of human cancer cachexia patients.

5.3.2 Cell Morphological Assessment: Myotube Number, Diameter and Area

Morphological differentiation was assessed using a cell imaging system at 10 x magnifications for representative images and morphological count/ analysis respectively (Leica, DMI 6000 B,

Milton Keynes, England, UK). Images were subsequently used for quantitative measures of myotube number and area. Light microscope images were imported into Image J software (Java software, National Institutes of Health, USA) for analyses. Myotube numbers were counted per image and global mean $\pm$ SD was determined across all images per experimental condition (a myotube was defined as containing 3 or more nuclei encapsulated within cellular structures, to avoid counting of cells undergoing mitosis). Myotube area (μm^2) was determined by carefully tracing around myotube structures after converting pixel length to μm using Image J software. All cell culture experiments were performed on 3 separate occasions ($n = 3$) and 3 images were taken per well and repeated across 2 wells per condition in each experiment (total of 18 images per condition). The $n = 3$ cells in this study were brought up from liquid nitrogen cryopreservation and all at the same passage number (passage number 5, however that had been frozen down on different dates).

5.3.3 Cell Extractions

At both at 30 minutes after dosing (noted as 0 hrs) and 72 hours following exposure to dosing conditions, cells were lysed in 300 μl /well TRIZOL reagent per well (Invitrogen Life Technologies, Carlsbad, Ca) and the RNA isolated and quantified as detailed below for downstream gene expression analysis by RT-PCR (detailed below).

5.3.4 RNA Extractions

Existing media was aspirated, cells were then washed briefly with PBS and lysed for 5 minutes at room temperature in 300 μl TRIZOL reagent per well of a 6-well plate (Invitrogen, Life Technologies, Paisley, UK.). Two wells of 6 well plates per condition were harvested using a cell scraper and then collected into 1.5 mL RNase free tubes per condition. Samples were agitated by vortexing for 10 seconds to ensure cells were totally lysed and were then stored at -20°C for up to 1 week. On the day of extraction homogenates were defrosted and 1 mL chloroform per 0.5 mL of TRIZOL reagent was added, prior to vigorous mixing for 15 seconds. Resulting mixtures were kept at room temperature for 10 minutes then centrifuged at 12000 g for 15 minutes at 4°C . Following centrifugation, the mixture separated into a lower red phenol-chloroform phase containing cell debris and components of TRIZOL, a milky-coloured interphase containing DNA and protein, and a colourless aqueous upper phase that housed the RNA and chloroform. The colourless aqueous phase was carefully extracted into new RNase free tubes, without disturbing the interphase. RNA was precipitated from the aqueous phase by mixing with isopropanol at a ratio of 1:1. Samples were stored at room temperature for 10

minutes and centrifuged at 12000 g for 10 minutes at 4°C. Supernatant was removed and the RNA pellet was washed by adding 1 mL of 75% ethanol. The samples were centrifuged at 7500 g for 8 minutes at 4°C. Ethanol was removed and the RNA pellet air-dried. RNA was dissolved in 20 µl RNA storage solution (Ambion-The RNA Company- Cheshire, UK) and vortexed for 10 seconds. All procedures above were carried out using RNA-free pipettes and tips and pipettes that were designated for RNA use only. A different tip was used for every sample to ensure no cross contamination. All work surfaces and equipment were sprayed with 70 % IMS and then RNase ZAP (Ambion-The RNA Company, Cheshire, UK) before commencing work. The samples were analysed immediately for RNA concentration.

5.3.5 RNA Concentration Analysis on the NANODROP Spectrophotometer

RNA purity and concentration was assessed using 1 µl of sample on a NanoDrop 2000c, UV-Vis (Ultraviolet–visible spectroscopy) spectrophotometer (Thermo Fisher Scientific, MA, USA) using ODs of 260 and 280 nm. A ratio of these OD value was calculated for each sample with all samples possessing 260/280 ratios and therefore accepted as high RNA quality.

5.3.6 Primer Design

Primer sequences (Table 5.0) were identified using Gene (NCBI, www.ncbi.nlm.nih.gov/gene) and designed using both web-based OligoPerfect™ Designer (Invitrogen, Carlsbad, CA, USA) and Primer-BLAST (NCBI, <http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Primers were purchased from Sigma (Suffolk, UK) without the requirement of further purification. Sequence homology (BLAST) searches ensured specificity to ensure the primers matched the sequence and therefore gene that they were designed for. Three or more GC bases in the last 5 bases at the 3' end of primers was avoided as stronger bonding of G and C bases can cause nonspecific amplification. Primer sequences were designed to exclude hairpins, self-dimer, and cross-dimers. All primers designed were amplified a product of between 76-280 bp (Table 5.0).

Table 5.0. Primer sequences for genes of interest.

Gene	Primer Sequence (5'-3')	Ref. Sequence number	Amplicon length (bp)
MyoD	F:CATTCCAACCCACAGAAC R:GGCGATAGAAGCTCCATA	NM_010866.2	125
Myogenin	F:CCAAGTGAAGATTGTCTGTC R:GGTGTTAGCCTTATGTAAT	NM_031189.2	173
IGF-I	F:GCTTGCTCACCTTTACCAGC R:TTTGGGCATGTCAGTGTGG	NM_001111276.1	280
IGFBP2	F:AGTGCCATCTCTTCTACAA R:GCTCAGTGGTTGGTCTCTT	NM_008342.3	197
IGFBP5	F:GACCCAGTCCAAGTTTGTGG R:GAATCCTTTGCGGTCACAGT	NM_010518.2	193
TNF- α	F:TCAACAACACTACTCAGAAACAC R:AGAACTCAGGAATGGACAT	NM_013693.3	130
TNFRS1a	F:CTGATCTCTATCTGCCTCTG R:CGCTCGTGAATGAAGTAAG	NM_001065.3	155
TNFRS1b	F:GTTGCTCTGTTATAGGATGGT R:TGCTGTCTGCTGTCTACT	NM_011610.3	113
MURF-1	F:CCAAGGAGAATAGCCACCAG R:CGCTCTTCTTCTCGTCCAG	NM_001039048.2	84
MAFBX-1	F:GTCGCAGCCAAGAAGAGAA R:CGAGAAGTCCAGTCTGTTGAA	NM_026346.3	156
Myostatin	F:TACTCCAGAATAGAAGCCATAA R:GTAGCGTGATAATCGTCATC	NM_010834.3	194
Fox01	F:AGTGGATGGTGAAGAGCGTG R:GAAGGGACAGATTGTGGCGA	NM_019739.3	96
Fox03	F:CGGACAAACGGCTCACTTT R:TCGGCTCTTGGTGTACTTG	NM_019740.2	272
NF- κ B	F:ACACGAGGCTACAACCTCTGC R:GGTACCCCCAGAGACCTCAT	NM_008689.2	164
p53	F:CATCCTGGCTGTAGGTAGCG R:GGCAGTCATCCAGTCTTCGG	NM_011640.3	109

5.3.7 RT-PCR and Analysis

RT-PCR was carried out using Quantifast SYBR green RT-PCR kit (Qiagen, Manchester, UK) on a Rotor-Gene® (Qiagen, Manchester, UK) supported by Rotor-Gene® Q Software, version 2.1.0.9 (Qiagen, Manchester, UK). The RT-PCR cycles consisted of; 48°C, 30 min (reverse transcription/ synthesis of cDNA), 95°C, 10 min (transcriptase inactivation and initial denaturation) followed by 40 cycles of 95°C, 15 sec (denaturation), 60°C, 1 min (annealing

and extension in 1 step). Disassociation melt-curve analysis was performed to reveal and therefore exclude non-specific amplification and primer dimer issues. All our gene products yielding a single melt peak/temperature suggesting one product was amplified. Relative gene expression analysis was carried out using $\Delta\Delta C_t$ equation, otherwise known as the Livak method (Schmittgen and Livak, 2008), this is to establish normalised expression ratios, where the relative expression is calculated as $2^{-\Delta\Delta C_t}$ and C_t represents the threshold cycle. RNA Polymerase II B (RPII β) was extremely stable between experimental conditions and therefore used as the housekeeping gene in all RT-PCR assays and the pooled mean used in the $\Delta\Delta C_t$ calculations. All RT-PCR figures are presented as a relative gene expression in comparison to the 0 hours DM control.

5.3.8 Statistical Analysis

Statistical analysis was carried out using the Statistical package for Social Science software program (SPSS V. 23, for Windows, Chicago, Illinois, USA). All Data was presented as means ($\pm$ SD). Two-way between subjects ANOVA (Condition [DM, N, TNF, and N+TNF] by Dose [31.25, 62.50 $\mu\text{g}\cdot\text{mL}^{-1}$]) were carried out for morphological and gene expression data. Post hoc tests were performed using Bonferroni. The results products through the Bonferroni tests are reported throughout the results as this test is more stringent.

5.4 Results

5.4.1 Myotube Number, Diameter and Area after nucleotide administration in the presence of TNF- α

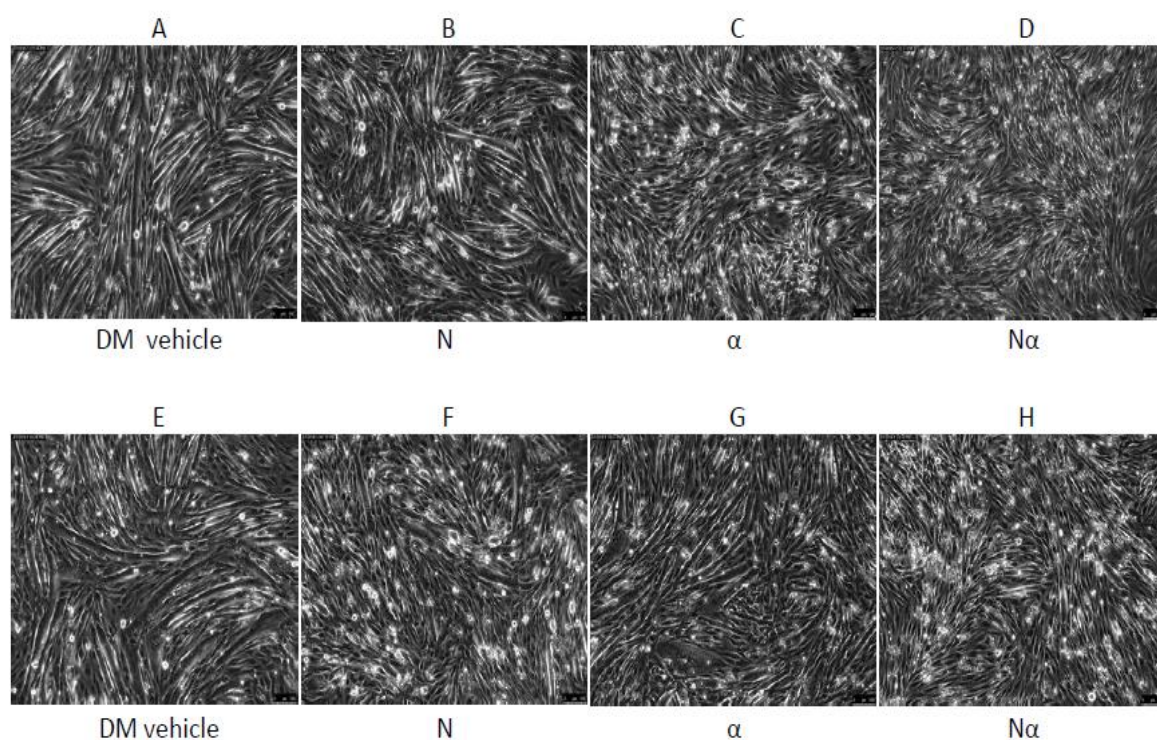


Figure 5.1.0. 10X magnification images representative morphological microscope images for all dosing conditions. Image A-D = DM vehicle, N = nucleotides at $31.25 \mu\text{g}\cdot\text{mL}^{-1}$, α = TNF- α ($20 \text{ ng}\cdot\text{mL}^{-1}$), N α = nucleotides at $31.25 \mu\text{g}\cdot\text{mL}^{-1}$ + TNF- α ($20 \text{ ng}\cdot\text{mL}^{-1}$). E-H = DM vehicle, N = nucleotides at $62.5 \mu\text{g}\cdot\text{mL}^{-1}$, α = TNF- α ($20 \text{ ng}\cdot\text{mL}^{-1}$), N α = nucleotides at $62.5 \mu\text{g}\cdot\text{mL}^{-1}$ + TNF- α ($20 \text{ ng}\cdot\text{mL}^{-1}$) for all conditions.

5.4.2 Myotube Number

For myotube number, a two-way ANOVA was conducted that examined the effect of dose (31.25 and 62.5 $\mu\text{g}\cdot\text{mL}^{-1}$) and treatment conditions at 7 days. The main effect for dose upon myotube number almost reached statistical significance ($F_{3,16} = 4.10$, $P = 0.06$). A significant main effect for treatment was apparent ($F_{3,16} = 6.54$, $P = 0.004$) with TNF- α and Nucleotides + TNF- α suppressed relative to DM and nucleotide treatments in 31.25 $\mu\text{g}\cdot\text{mL}^{-1}$ dose condition alone. Whilst reduced in the 62.5 $\mu\text{g}\cdot\text{mL}^{-1}$ dose there was not a significant treatment effect apparent reflecting the relative suppression of all treatment condition at this dose. There was no statistically significant interaction between dose and conditions ($F_{3,16} = 1.03$, $P = 0.40$).

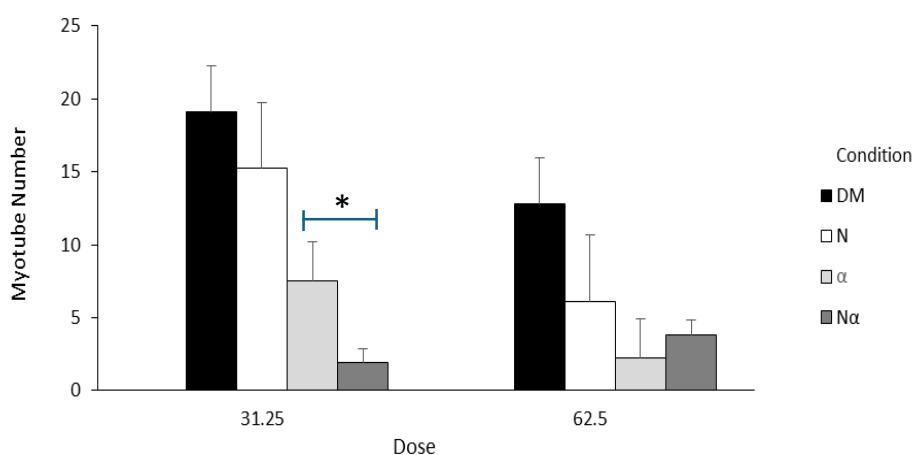


Figure 5.1.1. Myotube number at nucleotide doses 31.25 $\mu\text{g}\cdot\text{mL}^{-1}$ and 62.5 $\mu\text{g}\cdot\text{mL}^{-1}$ across dose and treatment conditions. Data are expressed as Mean $\pm$ SD values. Significantly reduced myotube number $P < 0.004$ which are denoted using a * between treatments.

5.4.3 Myotube Diameter

There was no main effect for dose upon myotube diameter ($F_{3, 16} = 2.94$, $P = 0.11$). However, there was a main effect for treatment condition with TNF- α as well as Nucleotides + TNF- α suppressed relative to DM and nucleotide treatments in $62.5 \mu\text{g}\cdot\text{mL}^{-1}$ dose conditions ($F_{3, 16} = 3.54$, $P = 0.04$). For myotube diameter, there was no statistically significant interaction between dose and conditions ($F_{3, 16} = 1.45$, $P = 0.27$).

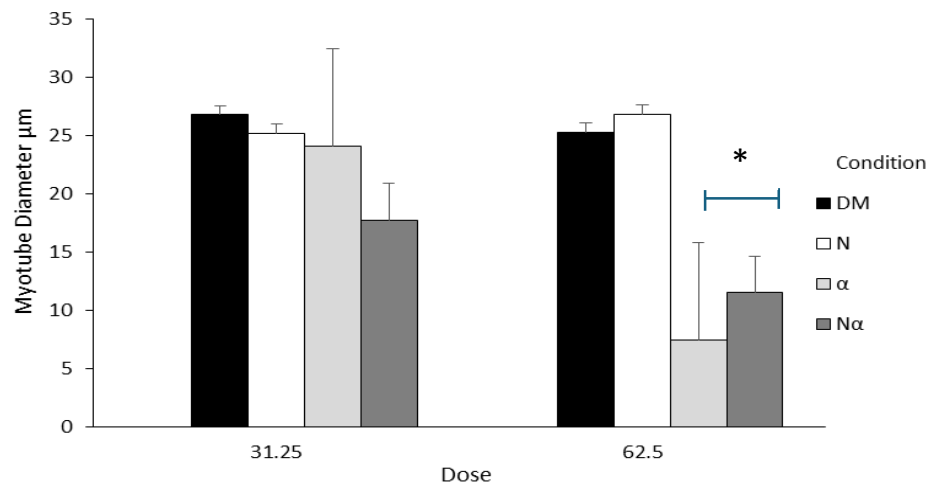


Figure 5.1.2. Myotube diameter at nucleotide doses $31.25 \mu\text{g}\cdot\text{mL}^{-1}$ and $62.5 \mu\text{g}\cdot\text{mL}^{-1}$ across treatments. Data are expressed as Mean $\pm$ SD values. Significant reduction expressed at $P < 0.05$.

5.4.4 Myotube Area

There was no main effect for dose upon myotube area ($F_{3, 16} = 2.88$, $P = 0.11$). However, there was a main effect for treatment condition with TNF- α as well as Nucleotides + TNF- α suppressed relative to DM and nucleotide treatments in $62.5 \mu\text{g}\cdot\text{mL}^{-1}$ dose condition ($F_{3, 16} = 5.09$, $P = 0.01$). For myotube area, there was no statistically significant interaction between dose and conditions ($F_{3, 16} = 2.00$, $P = 0.16$).

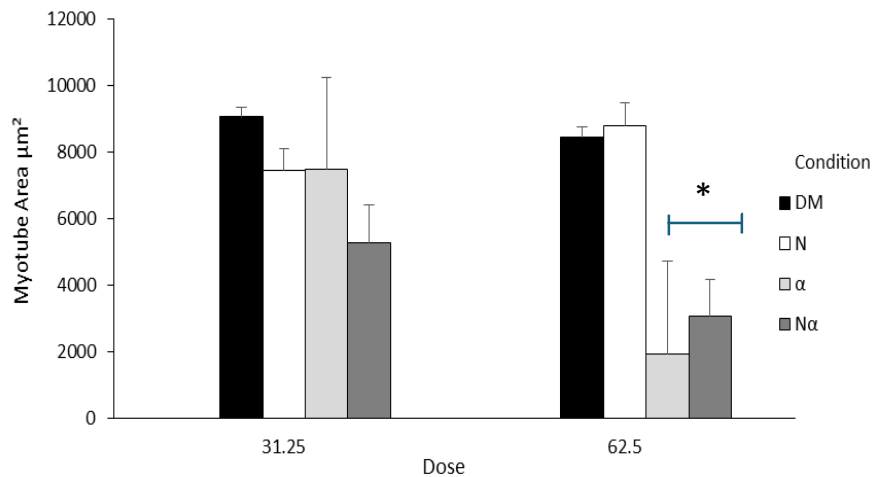


Figure 5.1.3. Myotube area at nucleotide doses $31.25 \mu\text{g}\cdot\text{mL}^{-1}$ and $62.5 \mu\text{g}\cdot\text{mL}^{-1}$ across conditions. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed at $P < 0.05$.

5.4.5 Gene Expression Data - Myogenesis Associated Genes: MyoD

There was a significant main effect for treatment alone ($F_{3, 40} = 25.01$, $P = 0.0001$), no significant main effect for dose was apparent ($F_{1, 40} = .275$, $P = 0.603$). There was no significant interaction between dose and condition ($F_{3, 40} = 2.38$, $P = 0.84$). (Figure 5.1.4), MyoD gene expression was significantly impaired in TNF- α treatment and Nucleotide + TNF- α treatment 31.25 and 62.5 $\mu\text{g}\cdot\text{mL}^{-1}$ relative to the DM and nucleotide treatments alone 31.25 and 62.5 $\mu\text{g}\cdot\text{mL}^{-1}$. No significant difference was observed between DM and nucleotide ($P \geq 0.05$).

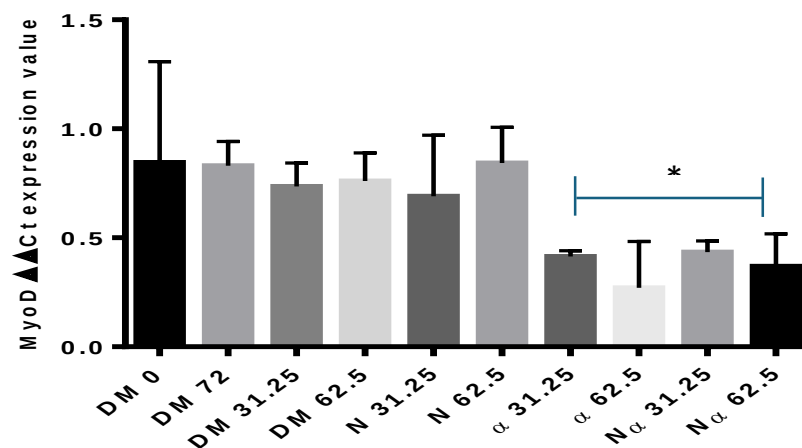


Figure 5.1.4. Gene expression data for MyoD across treatments and doses. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed at $P < 0.0001$ which is denoted using a *.

5.4.6 Gene Expression Data - Myogenesis Associated Genes: Myogenin

There was a significant main effect for treatment alone ($F_{3, 38} = 16.143$, $P < 0.001$), no significant main effect for dose was apparent ($F_{1, 38} = 1.193$, $P = 0.282$). There was no significant interaction between dose and condition treatment ($F_{3, 38} = 0.392$, $P = 0.759$). On average (Figure 5.1.5), myogenin gene expression across all treatments was lower relative to the Media + Vehicle treatment (DM 72) ($P < 0.001$). Myogenin expression relative to DM ($P < 0.01$) was impaired in TNF- α alone and Nucleotide +TNF- α treatments (31.25 and 62.5 $\mu\text{g}\cdot\text{mL}^{-1}$) no difference was observed between both TNF- α conditions.

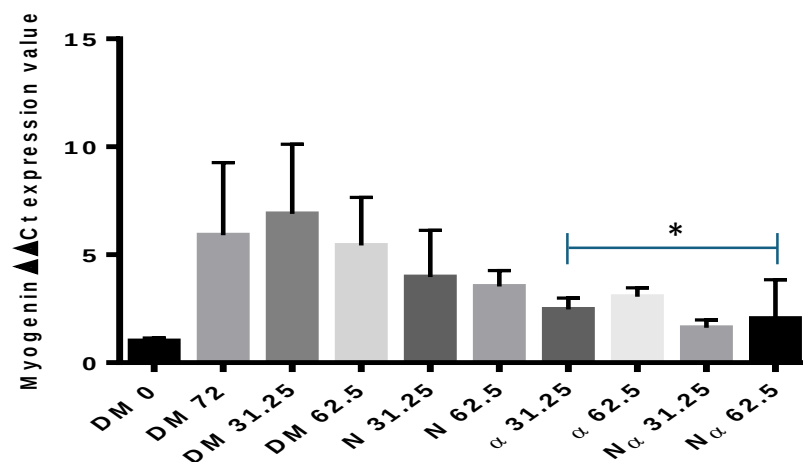


Figure 5.1.5. Gene expression data for Myogenin across treatments and doses. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed at $P < 0.001$ which is denoted using a *.

5.4.7 Gene Expression Data - Growth factors known to regulate differentiation: IGF-1

There was a significant main effect for treatment alone ($F_{3,40} = 9.373$, $P < 0.001$), no significant main effect for dose was apparent ($F_{1,40} = 0.02$, $P = 0.889$). There was no significant interaction between dose and condition, was evident ($F_{3,40} = 1.38$, $P = 0.262$). On average (Figure 5.1.6), IGF-1 gene expression was significantly impaired in the TNF- α alone and Nucleotide + TNF- α treatments relative to the DM and nucleotide treatments.

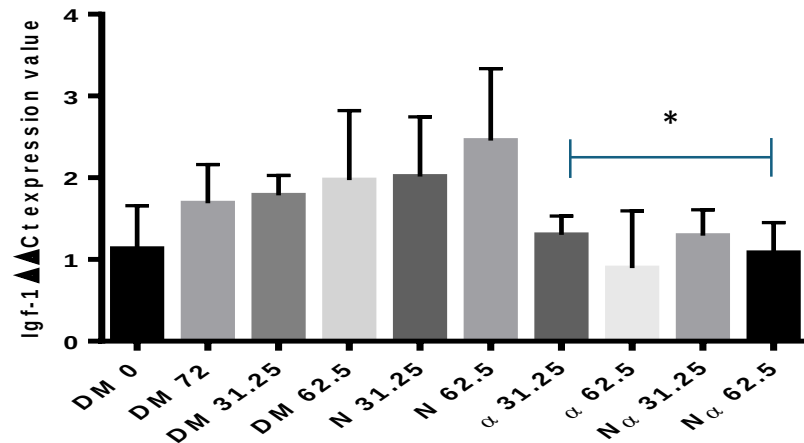


Figure 5.1.6. Gene expression data for IGF-1 across treatments and doses. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed at $P < 0.05$ which is denoted using a *.

5.4.8 Gene Expression Data - Growth factors known to regulate differentiation: IGFBP2

There was a significant main effect for treatment alone ($F_{3, 40} = 4.968$, $P = 0.005$), no significant main effect for dose was apparent ($F_{1, 40} = 0.501$, $P = 0.483$). There was no significant interaction between dose and condition ($F_{3, 40} = 1.53$, $P = 0.221$). (Figure 5.1.7), IGFBP2 gene expression was significantly higher in the nucleotide 62.5 treatment condition relative to DM ($P < 0.05$), and TNF- α alone ($P < 0.05$).

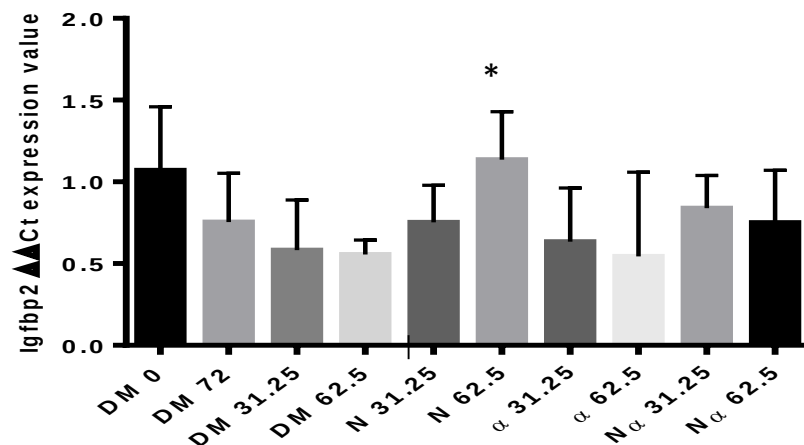


Figure 5.1.7. Gene expression data for IGFBP2 across treatments and doses. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed at $P < 0.005$ which is denoted using a *.

5.4.9 Gene Expression Data - Growth factors known to regulate differentiation: IGFBP5

There was a significant main effect for condition alone ($F_{3, 40} = 8.574$, $P < 0.001$), no significant main effect for dose was apparent ($F_{1, 40} = 2.432$, $P = 0.127$). There was no significant interaction between dose and condition ($F_{3, 40} = 0.542$, $P = 0.656$). (Figure 5.1.8), IGFBP5 gene expression was significantly impaired in the TNF- α alone and Nucleotide + TNF- α treatment relative to the treatment and nucleotide conditions. Nucleotides did not enhance expression relative to DM.

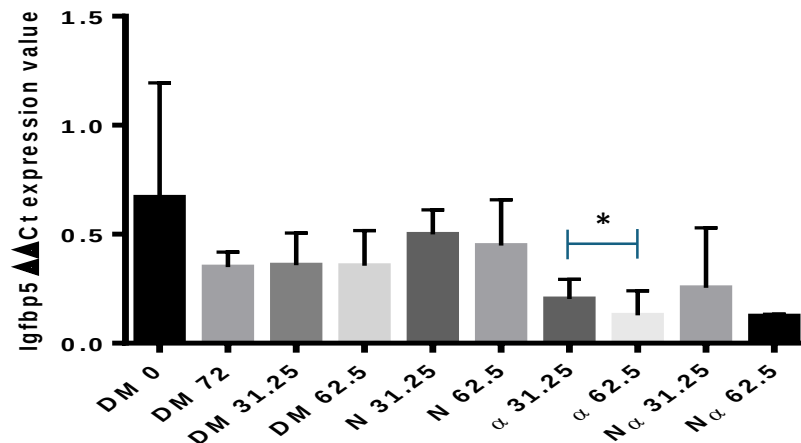


Figure 5.1.8. Gene expression data for IGFBP5 across treatments and doses. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed at $P < 0.0001$ which is denoted using a *.

5.4.10 Gene Expression Data - TNF- α signalling associated transcript

There was no significant main effect for condition ($F_{3, 40} = 2.526$, $P = 0.071$), no significant main effect for dose was apparent ($F_{1, 40} = 1.471$, $P = 0.232$). There was no significant interaction between dose and treatment ($F_{3, 40} = 2.083$, $P = 0.118$).

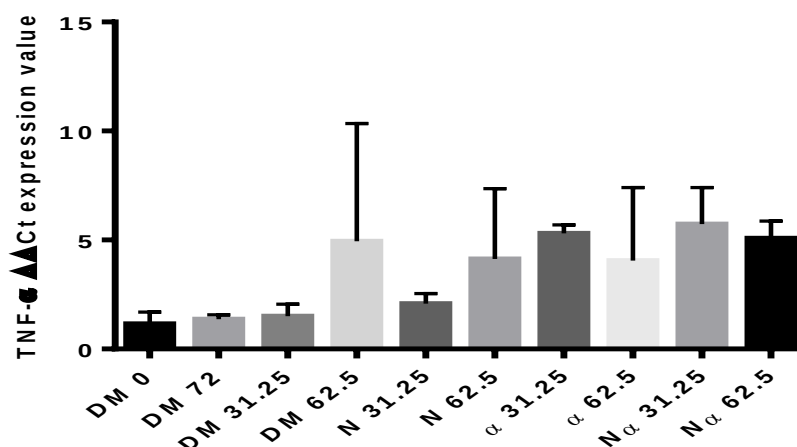


Figure 5.1.9. Gene expression data for TNF- α across treatments and doses. Data are expressed as Mean $\pm$ SD values. No significant difference was expressed.

5.4.11 Gene Expression Data – TNFRSF1a

There was no significant main effect for treatment ($F_{3, 40} = 2.65$, $P = 0.062$), no significant main effect for dose was apparent ($F_{1, 40} = 0.419$, $P = 0.521$). There was no significant interaction between dose and condition ($F_{3, 40} = 3.45$, $P = 0.25$). (Figure 5.2.0), TNFRSF1a gene expression increased in the high dose condition for both nucleotide and Nucleotide + TNF- α condition alone ($P \geq 0.05$).

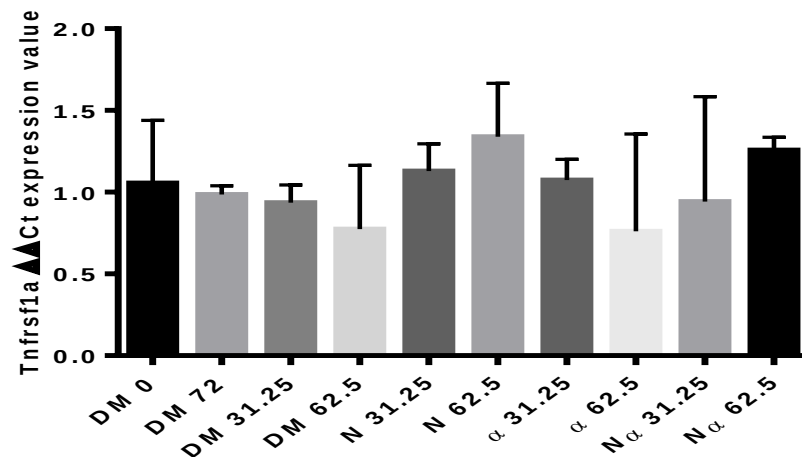


Figure 5.2.0. Gene expression data for TNFRSF1a across treatments and doses. Data are expressed as Mean $\pm$ SD values. No significant differences were apparent.

5.4.12 Gene Expression Data – TNFRSF1b

There was a significant main effect for condition alone ($F_{3, 40} = 5.390$, $P = 0.003$), no significant main effect for dose was apparent ($F_{1, 40} = 0.152$, $P = 0.698$). There was a small but significant interaction between dose and condition ($F_{3, 40} = 0.2886$, $P = 0.047$). (Figure 5.2.1), TNFRSF1b gene expression was significantly elevated in the TNF- α 31.25 treatment as well as Nucleotide + TNF- α relative to DM 31.25 treatment only.

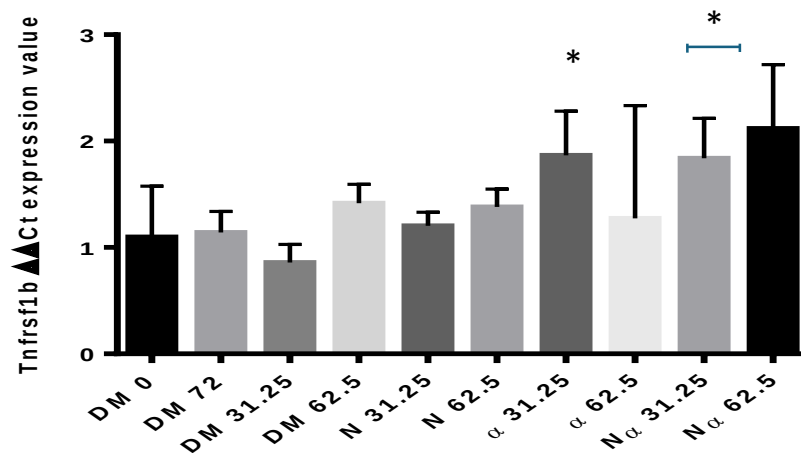


Figure 5.2.1. Gene expression data for TNFRSF1b across treatments and doses. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed at $P < 0.05$ which is denoted using a *.

5.4.13 Gene Expression Data - Genes associated with apoptosis and muscle atrophy: MURF-1

There was a significant main effect for condition ($F_{3, 40} = 14.53$, $P < 0.001$), a significant main effect for dose was also apparent ($F_{1, 40} = 6.74$, $P = 0.013$). There was no significant interaction between dose and condition ($F_{3, 40} = 1.44$, $P = 0.245$). (Figure 5.2.2), MURF-1 gene expression was significantly lower across the Nucleotide 62.5, both TNF- α treatments as well as Nucleotide + TNF- α relative to DM conditions.

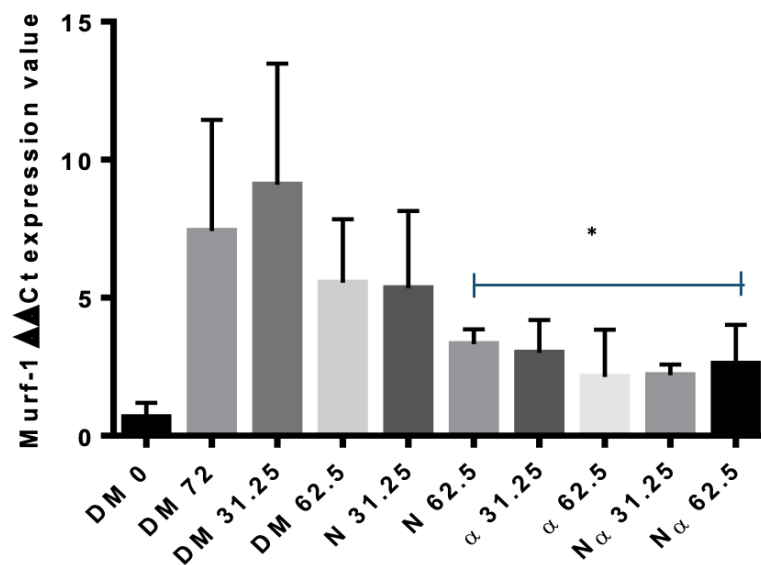


Figure 5.2.2. Gene expression data for MURF-1 across treatments and doses. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed at $P < 0.05$ which is denoted using a *.

5.4.14 Gene Expression Data - Genes associated with apoptosis and muscle atrophy: MAFBX-1.

There was no significant main effect for conditions alone ($F_{3, 40} = 1.973$, $P = 0.134$), no significant main effect for dose was apparent ($F_{1, 40} = 0.146$, $P = 0.704$). There was no significant interaction between dose and condition ($F_{3, 40} = 0.623$, $P = 0.604$) (Figure 5.2.3).

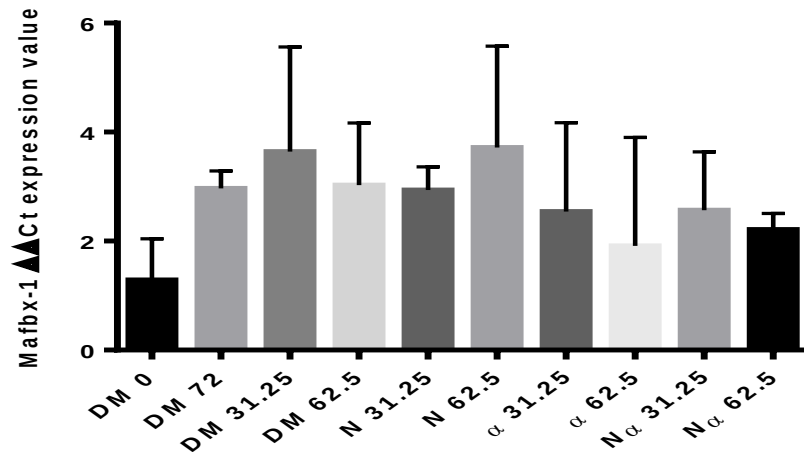


Figure 5.2.3. Gene expression data for Mafbx-1 across treatments and doses. Data are expressed as Mean $\pm$ SD values.

5.4.15 Gene Expression Data - Genes associated with apoptosis and muscle atrophy: Myostatin

There was no significant main effect for condition alone ($F_{3, 40} = 0.113$, $P = 0.952$), no significant main effect for dose was apparent ($F_{1, 40} = 2.308$, $P = 0.137$). There was no significant interaction between dose and ($F_{3, 40} = 1.76$, $P = 0.170$). (Figure 5.2.4)

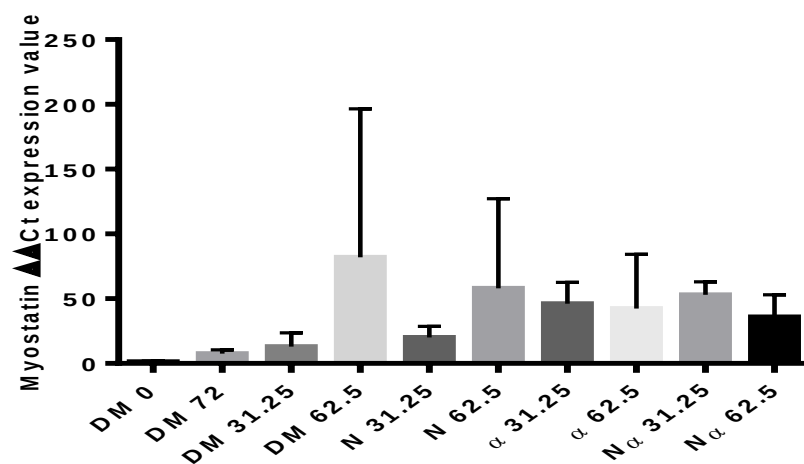


Figure 5.2.4. Gene expression data for Myostatin across treatments and doses. Data are expressed as Mean $\pm$ SD values.

5.4.16 Gene Expression Data - Genes associated with apoptosis and muscle atrophy: Fox01

There was a significant main effect for treatment alone ($F_{3, 40} = 24.55$, $P < 0.001$), no significant main effect for dose was apparent ($F_{1, 40} = 1.301$, $P = 0.261$). There was no significant interaction between dose and treatment ($F_{3, 40} = 1.77$, $P = 0.168$). (Figure 5.2.5), Fox01 gene expression was significantly reduced in the nucleotide + TNF- α and TNF- α alone relative to the DM condition. Nucleotide treatment did not differ from DM.

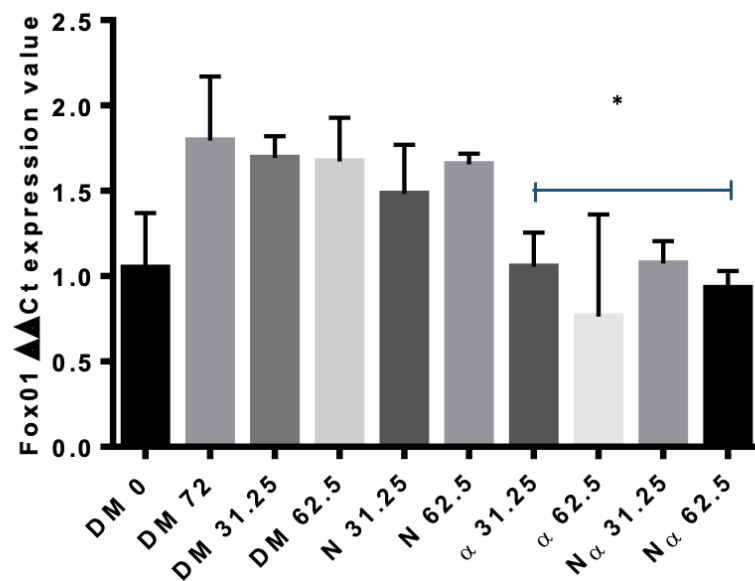


Figure 5.2.5. Gene expression data for Fox01 across treatments and doses. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed as $P < 0.001$ which is denoted using a *.

5.4.17 Gene Expression Data - Genes associated with apoptosis and muscle atrophy: Fox03

There was no significant main effect for condition alone ($F_{3, 40} = 0.103$, $P = 0.958$), no significant main effect for dose was apparent ($F_{1, 40} = 0.023$, $P = 0.879$). There was no significant interaction between dose and condition ($F_{3, 40} = 0.359$, $P = 0.783$) (Figure 5.2.6).

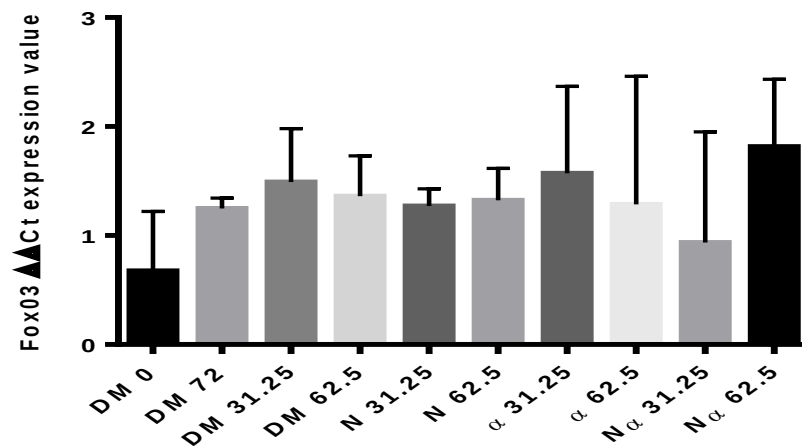


Figure 5.2.6. Gene expression data for Fox03 across treatment conditions. Data are expressed as Mean $\pm$ SD values.

5.4.18 Gene Expression Data - Genes associated with apoptosis and muscle atrophy: NF- κ B

There was no significant main effect for condition alone ($F_{3, 40} = 1.55$, $P = 0.216$), no significant main effect for dose was apparent ($F_{1, 40} = 0.03$, $P = 0.86$). There was no significant interaction between dose and condition ($F_{3, 40} = 1.92$, $P = 0.14$) (Figure 5.2.7).

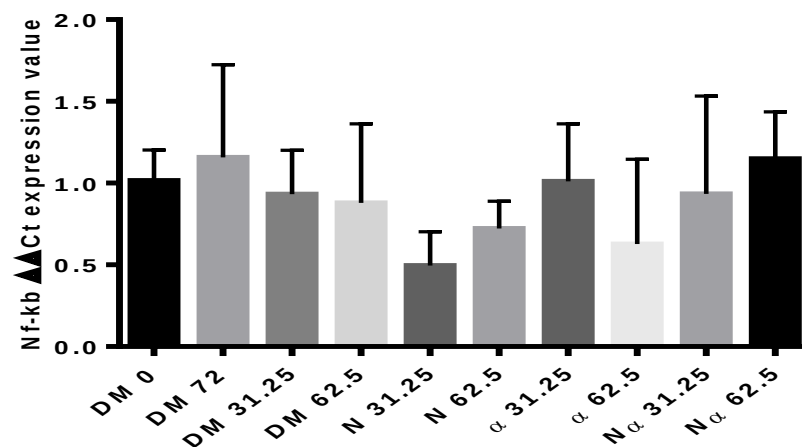


Figure 5.2.7. Gene expression data for NF- κ B across conditions. Data are expressed as Mean $\pm$ SD values.

5.4.19 Gene Expression Data - Genes associated with apoptosis and muscle atrophy: p53

There was a significant main effect for treatment alone ($F_{3, 40} = 3.11$, $P = 0.037$), no significant main effect for dose was apparent ($F_{1, 40} = 2.36$, $P = 0.132$). There was a significant interaction between dose and treatment ($F_{3, 40} = 7.04$, $P = 0.001$). TNF- α alone at 31.25 and Nucleotide + TNF- α at 62.5 relative to the DM and Nucleotide treatments was elevated. Nucleotide treatment did not differ from DM.

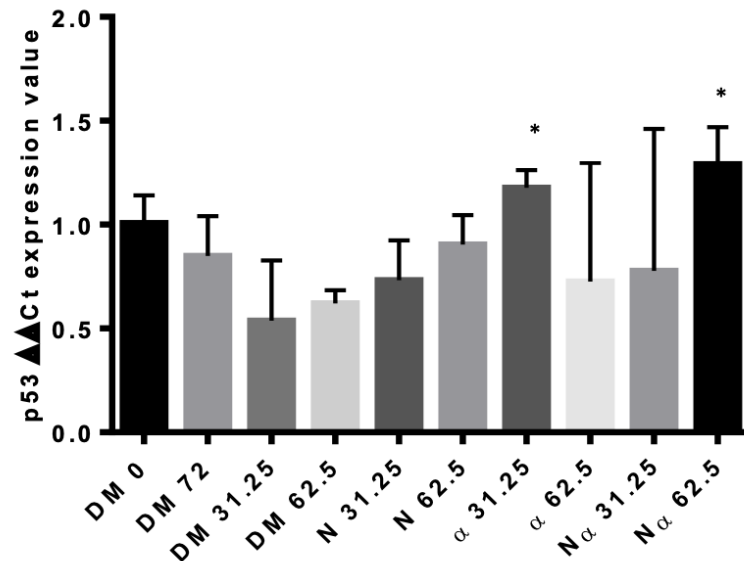


Figure 5.2.8 Gene expression data for p53 across conditions. Data are expressed as Mean $\pm$ SD values. Significant difference is expressed at $P < 0.05$ which is denoted using a *.

5.5 Discussion

Skeletal muscle is a striated tissue which plays a key role in regulating metabolism and homeostasis in the organism, accounting for ~40% of adult human body weight (Frontera and Ochala, 2015). Skeletal muscle myofibers originate from myogenic progenitor cells (MPCs), that which differentiate into myoblasts (Howard et al., 2020). These myoblasts subsequently undergo proliferation, differentiation, and fusion to form multinucleated myotubes, a process regulated by a complex network of myogenic regulatory factors, such as MyoD and myogenin. Additionally, MPC cells give rise to satellite cells (muscle stem cell), which reside between the sarcolemma and basal lamina of muscle fibers and are located to optimise skeletal muscle maintenance, repair, and regeneration (Liu et al., 2018; Relaix et al., 2021). Under normal conditions these remain in a quiescent state. However, in response to muscle damage, they are rapidly activated and re-initiate the cell cycle, differentiating into proliferating myoblasts. This process is driven by a complex cascade of myogenic regulatory factors (transcription factors) which serve as key regulators of differentiation, and growth to produce myotubes, which eventually develop into new muscle fibers restoring tissue morphology and function (Liu et al., 2023).

Nucleotides play a critical role in the regulation of cellular energy and protein homeostasis, cell signalling to facilitate repair, recovery, and repletion of tissue function across multiple tissue types including muscle (Hess and Greenberg 2012; Ding et al., 2021; Nakagawara et al., 2022). As such dietary nucleotide can be considered conditionally essential nutrients because they tend to be deficient under conditions of high stress such as tissue regeneration, repair and growth (Galla et al., 2008; Tie et al., 2021). The aim of this study was to investigate whether nucleotide supplementation in the presence of TNF- α could preserve myoblast differentiation and growth, preventing myotube atrophy while also determining changes in regulatory gene expression associated with these processes. It was hypothesised that nucleotides would serve as a regulator of TNF- α -induced inflammatory stress, attenuating its known detrimental effects on C₂C₁₂ myoblast differentiation and myotube growth, as observed in prior studies involving chronic TNF- α exposure (Sharples et al., 2010; Sharples et al., 2016; Girven et al., 2016).

In the present study, murine C₂C₁₂ myoblast cells were used to explore the effect of different nucleotide doses upon on myoblast differentiation, myotube fusion and growth in the presence and absence of an artificial stress model induced by the chronic elevation of TNF- α (Girven et al., 2016). Tumour Necrosis Factor-alpha (TNF- α) is a pro-inflammatory cytokine implicated

in a variety of cellular processes, including apoptosis, inflammation, and tissue remodelling (Fu et al., 2024). In the context of skeletal muscle, high doses of TNF- α have been shown to induce increase catabolism and myotoxicity, particularly on the C₂C₁₂ mouse myoblast cell line. High concentrations of TNF- α exposure can disrupt myogenesis by inhibiting the differentiation of myoblasts into myotubes, primarily through the activation of the nuclear factor-kappa B (NF- κ B) signalling pathway (Alvarez et al., 2024). Additionally, high doses of TNF- α can induce oxidative stress, promote mitochondrial dysfunction, and upregulate the expression of muscle-specific atrophy factors such as MuRF1 and Mafbx (Atrogin-1), leading to increased apoptosis related muscle atrophy (Jang et al., 2021; Li et al., 2023).

All these pathways are susceptible to nucleotides intervening as a potential countermeasure (Ding et al., 2021; Nakagawara et al., 2022; Nakagawara et al., 2023; Zhu et al., 2023; Wu 2024). However, despite these considerations, the initial hypothesis proposed in this chapter, was rejected, in that nucleotides did not offset the adverse effects of TNF- α on myoblast differentiation, growth and myotube morphology. Data indicated that myoblast differentiation and growth proceeded as expected in the control conditions (DM), although as will be discussed below an effect of the cell membrane permeabilisation agent (DMSO) may have been expressed that reduced myotube formation were present at higher concentrations. The morphological data broadly indicate relative to control (DM) that the presence of TNF- α induced significant atrophy expressed as reductions in myotube number, diameter, and area). Myotube number declined in a step wise manner in the presence of nucleotides rather than the expected increase. Similarly, the co-incubation of C₂C₁₂ with TNF- α and nucleotide rather than reversing these effects, evoked further reductions in myotube number, diameter, and area to a greater extent than TNF- α alone treatments. These findings correspond with previous studies, whereby chronic TNF- α administration reduces differentiation in myoblasts and in myotube resulting in atrophy (Sharples et al., 2010; Myer et al., 2015; Girven et al., 2016). The effects of TNF- α on myogenesis are concentration dependent. Higher concentrations of TNF- α (10-20 ng·mL⁻¹) inhibit the progression of myogenesis and impair proliferation and differentiation of cells in vitro (Girven et al., 2016; Howard et al., 2020). The decline in myotube numbers, diameter and area broadly indicated a potential combined effect between nucleotide and TNF- α that accentuated rather than attenuated the impairment of cell proliferation (Figure 5.1.1). Differentiation of myoblasts into myotubes and their subsequent development was not enhanced by the presence of nucleotides. Irrespective of nucleotide dose it broadly did not elevate cell morphology and/or gene transcript data. When added as a countermeasure into the

TNF- α stress model, the proposed rescue of myotube growth and attenuation in atrophy was not expressed.

These observations are broadly contrary to the reported *in vivo* and *in vitro* effects of nucleotide and nucleotide precursor (glutamine) administration upon skeletal muscle growth (Girven et al., 2016; Tie et al., 2021; Wang et al., 2024). *In vivo*, the supplementation of animal diets with nucleotides improves skeletal muscle growth performance, enhances carcass yield, and supports overall animal health (Fiona et al., 2019; Bonagurio et al., 2022). The availability of nucleotides can be rate-limiting in rapidly dividing tissues, particularly under conditions of high stress (Ding et al., 2021; Gopi et al., 2023). Exogenous nucleotide supplementation not only overcomes this limitation but also reduces the animal's energy expenditure, as *de novo* nucleotide synthesis is an energy-demanding process. Bypassing the *de novo* biosynthesis pathway of nucleotides *in vivo* can be used to conserve energy and reduce the high energy consumption of *de novo* nucleotide synthesis so that more energy can be invested into growth (Li and Gatlin, 2006). This is particularly critical during growth phases and reproductive periods (Bonato, 2017; Bonagurio et al., 2022). Dietary nucleotide supplementation therefore promotes higher body weight and daily weight gains through increased muscle growth rates and deposition, in bovine, porcine, poultry, and aquaculture species (Krol, 2011; Hess and Greenberg, 2012; Ding et al., 2021; Wang et al., 2024). Collectively, these benefits translate into higher skeletal muscle yields and improved meat quality in animal husbandry highlighting the application of dietary nucleotides in these production systems (Upadhyay and Vishwa, 2014; Tie et al., 2019; Aziz et al., 2024). The observed improvements in carcass quality are directly attributed to the role nucleotides play in elevating protein synthesis and cellular proliferation, both of which are critical for muscle development (Hossain et al., 2020; El-Nokrashy et al., 2021; Kamel et al., 2021). Miller et al. (2019) has demonstrated in an atrophic skeletal muscle model that atrophy is associated with reduced adenine nucleotide content in muscle primarily due to an increased rate of degradation, driven by elevated expression and activity of adenine-degrading enzymes. More recently they suggest that reducing purine degradation or elevating purine supply in muscle could be a viable approach to attenuate muscle tissue atrophy (Miller et al., 2024). In humans, Inoue et al. (2024) has indicated that the nucleotide uridine 5'-monophosphate (5'-UMP) delivered orally for 2 weeks can counteract disuse-induced muscle atrophy during a detraining stimulus.

These observed improvements in whole-body muscle gain and carcass yield may be mechanistically linked to nucleotide modulated cellular processes (Hossain et al., 2020; Wang et al., 2024). Recent in vitro studies using C₂C₁₂ myoblast cells have demonstrated that nucleotides can enhance myogenesis by promoting myoblast proliferation, differentiation, and growth. Uberti et al. (2020) reported that, in a caffeine-induced C₂C₁₂ stress model, nucleotides improved cell growth and differentiation, although the outcomes varied depending upon the exact dose and formulation of the proprietary nucleotides used. Nakagawara et al. (2022) showed that both cytidine-5'-monophosphate (5'-CMP) and uridine-5'-monophosphate (5'-UMP), along with their nucleoside precursors cytidine and uridine, promoted myogenic differentiation, myotube formation, and mitochondrial biogenesis through upregulation of PGC-1 α . Similarly, Nakagawara et al. (2023) demonstrated that muscle atrophy in a dexamethasone-mediated C₂C₁₂ model was reduced through a comparable pathway. Notably, these studies indicated that the myogenic effects of nucleotides were enhanced to a greater extent when pyrimidines were delivered as nucleosides (cytidine and uridine) rather than as nucleotides (5'-CMP and 5'-UMP). These findings suggest that both nucleoside and nucleotide forms play a role in muscle repair by promoting differentiation and mitigating atrophy potentially as a function of improved cellular uptake. Sawano et al. (2020) observed that a beef extract, containing high but undefined concentrations of nucleotides, significantly increased C₂C₁₂ myoblast proliferation, myotube diameter, and protein synthesis in a dose-dependent manner; however, excessive concentrations inhibited these effects. Similarly, Girven et al. (2016) demonstrated that L-glutamine, a precursor for de novo nucleotide synthesis, restored myoblast differentiation in the presence of TNF- α and confirmed its role in rescuing myotube atrophy in TNF- α -treated myotubes. However, the present study did not observe this rescue effect when nucleotides were provided in the TNF- α model. Across treatment conditions increasing nucleotide supply failed to attenuate atrophy and maintain/improve cell growth relative to the control conditions; in our hands present data is unsupportive of the current nucleotide formulation and dose regimen. This discrepancy may result from suboptimal nucleotide formulations and/or dosages that were insufficient to counteract the specific TNF- α toxicity model used. The nucleotide formulation used in this study was a proprietary mix of purine and pyrimidine nucleotides, with a balance favouring pyrimidines, including cytosine, uridine, thymidine, adenosine, and guanine. The absence of similar positive effects upon cell growth to those reported by Nakagawara et al. (2022) remains unclear, particularly as the relative amounts of 5'-CMP and 5'-UMP delivered in this study cannot be directly compared to their work. Xu et al. (2017) and Uberti et al. (2020) observed distinct adaptations in

mitochondrial enzyme concentrations, energy metabolism, and antioxidant responses in both rodent exercise models and C₂C₁₂ myotube models, respectively, depending on the specific nucleotide formulation used. As suggested by Uberti et al. (2020) the formulation administered is critical, with the nucleotide type and dose impacting cell growth differently. Previous studies in animal and aquaculture models have reported that supplementation with individual purine or pyrimidine nucleotides, as opposed to mixed formulations, can result in greater improvements in growth and stress tolerance (Song et al., 2012; Hossain, 2016; Hossain, 2020). Similarly, Guo et al. (2016) and Mok et al. (2019) demonstrated that individual nucleotide supplementation was more effective than mixed formulations. However, the present experimental design does not allow for the separation of these effects. Future studies should adopt more comprehensive models to evaluate the relative contributions of specific nucleotide formulations to myoblast differentiation, growth and myotube development. Furthermore, inappropriate nucleotide dose/concentrations administered in vitro may fail to support proliferation and growth but could also impair these processes (Sawano et al., 2020; Wang et al., 2024). Defining optimal doses and formulations for nucleotide administration remains challenging. While the present study undertook dose-response work and indicated that the applied nucleotide dosing concentrations were non-toxic (no cell death was observed), the viability assay used may lack the sensitivity to detect subtle functional impairments in processes such as cell signalling or energy metabolism, which could affect cell growth/atrophy.

In the present study, the cell differentiation media was supplemented with the nucleotide formulation as a delivery approach. However, the anticipated improvements in myoblast/myotube growth and differentiation were not observed, failing to replicate previously reported findings in C₂C₁₂ cell lines (Uberti et al., 2020; Nakagawara et al., 2022; Nakagawara et al., 2023). These discrepancies, notwithstanding the points around dose and formulation emphasise the critical role of bioavailability in influencing cell differentiation and growth. Support for this idea may be taken from the observations of Nakagawara et al. (2022) and Nakagawara et al. (2023), who demonstrate improved C₂C₁₂ myoblast and myotube growth and differentiation, in the presence of both nucleosides and nucleotides relative to control. However, nucleosides showed enhanced performance relative to the nucleotides. A key factor in this observation may lie in the differences in cellular transport and uptake mechanisms for nucleosides and nucleotides. Nucleoside transporters selectively facilitate nucleoside uptake based on cellular demand, owing to differences in their size, charge, and structure. In contrast, nucleotides, which are larger, more polar, and possess a phosphate group, cannot cross the

phospholipid cell membranes without specialised transport systems that are limited and need to be converted to nucleosides to transit the cell membrane (Vieites et al., 2008; Rodríguez-Serrano et al., 2010). These differences in transport likely contribute to bioavailability mediated variation in the efficacy observed between nucleotides and nucleosides (Nakagawara et al., 2022; Nakagawara et al., 2023).

In the present study, C₂C₁₂ myoblasts were incubated with nucleotides directly in the differentiation media and as such may present a barrier to bioavailability. While it is plausible that foetal calf serum (FCS) and foetal bovine serum (FBS) in the media contain enzymes such as 5'-nucleotidases, nucleosidases, and nucleoside phosphorylases, which can degrade nucleotides into nucleosides and enhance their bioavailability, the quantities of these enzymes are likely insufficient to induce meaningful changes in intracellular nucleotide concentrations capable of affecting myoblast and myotube growth. To enhance bioavailability, the nucleotide formulations (31.25 µg·mL⁻¹ and 62.5 µg·mL⁻¹) were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 0.02% and 0.04%, respectively. DMSO, an organic polar solvent, is widely used to solubilise poorly soluble molecules and facilitate their permeability across lipid membranes. At concentrations below 10%, DMSO is considered nontoxic; Schmidt et al. (1989) reported no adverse effects on cell growth at 1.4% DMSO, although this has been challenged in more recent studies (Awan et al., 2020). Notably, several studies have demonstrated that even low concentrations of DMSO can significantly affect C₂C₁₂ cell behaviour. For example, DMSO concentrations as low as 0.5% can impair cell attachment, viability, myogenesis, and morphological development in a dose-dependent manner (Orzechowski et al., 2001; Pal et al., 2012; Awan et al., 2020). To minimise these effects, the maximum DMSO concentration in this study was tested and limited to 0.04%. Nonetheless, the dose-response toxicity analysis revealed that while these concentrations did not result in extensive cell death, they did not fully eliminate cytotoxicity. This may explain the observed differences in myotube formation (Figure 5.1.1) between low and high nucleotide doses, where the higher DMSO concentration could have exacerbated potential adverse effects. Moreover, while the concentrations used avoided widespread cell death, they did not account for any potential impacts on gene expression. Previous research has demonstrated that even low DMSO concentrations can alter gene regulation. For instance, Nishimura et al. (2008) reported concentrations as low as 0.02% were found to upregulate atrophy-related genes, such as *Fox03a* and *MURF-1*. Similarly, Verheijen et al. (2019) demonstrated that DMSO concentrations below 0.02% could alter phenotypic characteristics, gene expression profiles,

protein content, and functional outcomes in differentiated cardiac myocytes. Taken together, these findings suggest that the approach used to solubilise nucleotides, while necessary for uptake, may have inadvertently impacted cell development and gene expression. This unintended interference may explain the atypical gene expression patterns observed in the mRNA data presented here in this chapter. Future studies should explore alternative solubilisation methods or nucleotide delivery strategies to minimize the potential confounding effects of DMSO.

Gene mRNA expression serves as the initial step in muscle differentiation and growth. During this process, transcription factors regulate the expression of genes essential for myotube formation and maturation (Sartori et al., 2021). The translated proteins resulting from mRNA changes subsequently drive cellular processes, expressed as morphological changes in the C₂C₁₂ cell line, transitioning from myoblast seeding through differentiation and maturation (Sartori et al., 2021). Thus, mRNA expression precedes and regulates protein synthesis required to produce the structural and functional changes observed during muscle differentiation and growth (Liu et al., 2023). Present data clearly demonstrate this process, as evidenced by differentiated and formed myotubes under different treatment and nutritional countermeasure C₂C₁₂ culture conditions. To understand the transcriptional regulation of this process of myoblast/myotube differentiation and growth in these C₂C₁₂ myoblast and myotubes under the inflammatory stress model. Selected molecular regulators critical for growth, differentiation, and maturation were assessed: growth factors regulating differentiation (IGF-I, IGFBP2, IGFBP5) and transcripts associated with TNF- α signalling, apoptosis, and muscle atrophy (TNF- α , MURF-1, MAFBX-1, Myostatin, FoxO1, FoxO3) were quantified. Apoptosis and cell survival-related markers (TNFRSF1a, TNFRSF1b, NF- κ B, p53) were quantified.

The expression of critical transcripts for myotube maturation and growth, including MyoD and Myogenin were undertaken. These transcription factors, both members of the myogenic regulatory factor (MRF) family, are essential for terminal differentiation and fusion of myoblasts into mature myotubes with MyoD functioning upstream to Myogenin (Li et al., 2023; Alvarez et al., 2024). Consistent with prior studies, chronic administration of C₂C₁₂ myotubes with TNF- α significantly suppressed MyoD and Myogenin mRNA transcripts relative to the differentiation media (DM) controls (Figure 5.1.4 and Figure 5.1.5), highlighting high dose chronic TNF- α 's established role as a suppressor of muscle differentiation and

supporting the efficacy of the model as a disruptor of normal cell growth (Sharples et al., 2010; Girven et al., 2016).

As a potential countermeasure, nucleotide co-administration was explored at two concentrations. Contrary to expectations, the combination of nucleotides and TNF- α further suppressed MyoD and Myogenin levels below those observed with TNF- α alone. This suggests a potential interaction wherein nucleotides in some manner exacerbate TNF- α 's anti-myogenic effects. In contrast, studies by Nakagawara et al. (2022), Nakagawara et al. (2023) and Wang et al. (2024) have reported that specific nucleotides, such as 5'-CMP and 5'-UMP, or broad spectrum purine and pyrimidine fed mix increases Myogenin mRNA expression, promoted myoblast differentiation, muscle protein synthesis, PGC1- α expression, increased mitochondrial DNA copy number and enhanced for myosin heavy chains mRNA expression along with enhanced structural integrity of myotube/muscle in vitro and in vivo. These discrepancies between the present work and these others may reflect differences between the in vitro stress induction model used. While the present study used TNF- α to induce chronic inflammatory stress, elevate previous studies employed other stressors like dexamethasone (Nakagawara et al., 2023) and hydrogen peroxide (Wu et al., 2024) to examine nucleotide efficacy in offsetting myotube atrophy and growth restriction. Mechanistic differences between these models, including the inhibition of anabolic signalling (PI3K/Akt/mTOR pathway by dexamethasone) versus the activation of NF- κ B and JNK pathways by TNF- α , may explain the divergent outcomes.

The observed reductions in MyoD and Myogenin with nucleotide supplementation could also reflect nucleotide dose-dependent effects, as excessive nucleotide delivered intracellularly in an unregulated manner through solubilisation of the plasma membrane with DMSO may disrupt uptake and thus cellular homeostasis, activating stress responses such as the tumour suppressor protein p53, which can inhibit differentiation, promote cell cycle arrest, atrophy or cell death (Diehl et al., 2022). P53 plays a key role in monitoring nucleotide pools during cellular stress, imbalances in nucleotide supply relative to demand can occur in terms of the nucleotide dose administered or inappropriate ratio of purine to pyrimidines nucleotides (Diehl et al., 2022). In the present work P53 was upregulated in the TNF- α and the high dose nucleotide + TNF- α treatment which may suggest such a mechanism may be at work in sensing both TNF- α treatment model and nucleotide-imposed stresses and may point toward a reason

for the synergism in suppressing MyoD and Myogenin. Support may come from the observations of Nakagawara et al. (2023) who indicate that depending on the nucleotide.

Several transcripts related to growth and atrophy were also considered. IGF-1 (Insulin-like Growth Factor-1), a key promoter of muscle hypertrophy and regeneration, by stimulating protein synthesis and inhibiting protein degradation along with its binding proteins IGFBP2 (Insulin-like Growth Factor Binding Protein 2) and IGFBP5 (Insulin-like Growth Factor Binding Protein 5) which acts as a regulatory factor for IGF-I activity by binding to regulate IGF-1 availability in tissues. Together, a significant reduction in IGF-1, and IGFBP5 were observed with a non-significant fall in IGFBP2 when cells were treated with prolonged TNF- α exposure and nucleotide + TNF- α relative to DM controls. These reductions align with IGF-1 and associated binding proteins changes reported by Girven et al. (2016), who demonstrated the adverse impact of TNF- α on muscle differentiation and myotube growth. Importantly, when IGF-1 is present it acts to inhibit NF- κ B and restore the fact that IGF-1 mRNA is down regulated suggest nucleotide not able to counteract this element of imposed cellular stress. These IGF family gene expression changes are consistent with gene expression down regulation seen in C₂C₁₂ myoblast differentiation and reduced myotube numbers reported by others (Foulstone et al., 2001; Sharples et al., 2013).

Additionally, genes associated with atrophy and muscle protein degradation, including MuRF-1 and Mafbx (Atrogin-1), are typically upregulated by TNF- α via pathways such as NF- κ B, Fox01 (Forkhead Box Protein 01) and Fox03 (Forkhead Box Protein 03) and myostatin. However, in the current study, the expression of NF- κ B, Fox01 and Fox03 genes showed no coherent overall expression changes either showing as unchanged or downregulated relative to DM in both the TNF- α and nucleotide + TNF- α treatment, contradicting expectations that TNF would significantly elevate their expression as reported by others (Sharples et al., 2010; Girven et al., 2016) and the morphological observations of atrophy i.e. decreased myotube diameter and area (Figure 5.1.2 and 5.1.3). Notably, MURF-1 was the only atrophy-related gene to show significant changes, but its expression was suppressed rather than increased. In contrast, provision of the nucleotides 5'-CMP and 5'-UMP alleviate but do not fully reverse dexamethasone-induced C₂C₁₂ related atrophy (Nakagawara et al., 2023). This being achieved through the regulation of MURF-1 and Mafbx expression; specifically, 5'-UMP decreased MURF-1 and MAFBX (Atrogin-1) mRNA levels that were upregulated by dexamethasone treatment in C₂C₁₂ myotubes.

This inconsistency may reflect the involvement of redundant or unmeasured signalling pathways, as TNF- α -induced NF- κ B activation typically upregulates atrophy genes (Girven et al., 2016). Furthermore, myostatin, an inhibitor of muscle growth through inhibition of satellite cell activation and proliferation, showed no significant expression changes, mirroring the results of Girven et al. (2016) but diverging from other studies reporting increased myostatin under TNF- α exposure (Karin et al., 2001).

An interesting finding was the increased expression of TNFRSF1b in TNF- α and nucleotide co-treatment groups. TNFRSF1b, which encodes a TNF receptor, may act as counter regulatory mechanism to TNF- α activity by binding the cytokine and reducing its bioavailability. Previous work by Girven et al. (2016) demonstrated that L-glutamine, a nucleotide precursor of the de novo synthesis pathways, significantly upregulated TNFRSF1b expression, potentially mitigating TNF- α 's detrimental effect upon cell atrophy. However, whether nucleotides directly modulate TNFRSF1b expression remains unclear and warrants further investigation. It is worth noting that no significant differences across treatments were determined in these remaining genes including those previously associated with high fold changes during the chronic administration of TNF- α as a stress induction model: Nf- κ B, TNF- α , TNFRSF1a, myostatin, MAFBX-1 and FoxO3.

The present study was limited to a small selection of mRNA transcripts, which may not fully capture the complex gene expression changes induced by TNF- α . The presence of DMSO as a potential confounder of gene expression should be acknowledged. Alvarez et al. (2024) analysed the influence of TNF- α at a high concentration (10 ng·mL⁻¹) on gene expression in myoblast C₂C₁₂ cells. They noted that even at this lesser concentration relative to the 20 ng·mL⁻¹ in the present work, treatment of C₂C₁₂ cells led to 954 differentially expressed genes compared to the controls with 326 upregulated and 628 downregulated after TNF- α incubation. The more limited pre-selected target and their subsequent inconclusive response is a methodological weakness of this chapter data set. Gene array transcripts analysis offers a more comprehensive approach to determining gene expression changes, providing a more global view of transcriptional responses compared to the limited mRNA analysis, which focuses only on a small pre-selected pool of genes as in the present work. It may be that a broader picture is required to understand gene expression changes following nucleotide provision given their role across potentially many signalling pathways and specificity of action different nucleotides may exert either specifically or synergistically across a range of cell types (Tie et al., 202 X;

Ding et al., 2021). The data is also limited, in that the translational protein expression was undetermined. Whilst mRNA transcripts can express multiple many fold changes either being up or down regulated unless protein resulting from that expression is produced then limited biological effects may ensue. Additionally, the observed disconnect between transcriptional and morphological outcomes suggests that further investigation is needed to understand the signalling cascades involved. For example, NF- κ B activity was anticipated to be elevated under TNF- α treatment but remained unchanged, potentially reflecting compensatory mechanisms or alternate pathways not measured in this study.

5.6 Conclusions

In conclusion, the effects of TNF- α on gene expression and myotube morphology in C₂C₁₂ cells highlight the complex interplay between inflammatory stress and muscle differentiation. While nucleotide supplementation was hypothesised to mitigate TNF- α 's catabolic effects, the current data suggest otherwise, with no clear transcriptional or morphological benefits observed. The present data set is not supportive of this current Nucleotide formulation and dose as a means of providing a counter measure to chronic TNF- α induced inflammation. These findings are somewhat at odds with several recent positive trials into the efficacy of nucleotides in a similar C₂C₁₂ model, albeit with different nucleotide formulations and concentrations. These findings confirm the need for further research into the molecular mechanisms governing muscle differentiation under inflammatory stress and the potential for targeted nucleotide nutritional interventions to provide support for these processes.

Chapter 6

Synthesis of Findings

This chapter serves to synthesise the key insights developed in this thesis regarding the interaction between nucleotide supplementation and physiological responses to high intensity exercise. Drawing from both in vivo and in vitro models, the findings collectively explored whether nucleotides function as ergogenic and /or adaptogenic agents capable of enhancing performance supporting gastrointestinal integrity, immune function, and cellular recovery under exercise and cytokine induced stress. Early chapters examined potential ergogenic effects during repeated sprint activity (RSA), suggesting limited outcomes. Subsequent investigations directed focus toward gastrointestinal resilience and immune modulation, drawing from gut permeability, damage and symptomology and contact hypersensitivity models. In the final experimental chapter, a shift to in vitro C₂C₁₂ myoblasts under TNF- α -induced inflammation, provided a more controlled and robust platform for evaluating nucleotide effects on cellular growth. Integrating these varied lines of evidence, this synthesis aims to clarify the contextual factors in this work that influenced the lack of observed nucleotide supplementation effectiveness. Together, these chapters illustrate the context-dependent efficacy of nucleotides, highlighting their role not as direct performance enhancers but as possible adaptogenic agents that support physiological systems. The findings will underscore the importance of model selection when assessing functional nutrients and point toward future research focused on stress intensity and targeted physiological outcomes. This integrated perspective reframes nucleotide supplementation within a broader paradigm of resilience in future demanding exercise stress conditions.

6.1 Fulfilment of aims and objectives

The primary aim of this chapter was to integrate and interpret the findings of each experimental chapter in relation to the original aim (s) and objectives of the thesis, set out in Chapter 1. A general discussion is then presented where specific attention is given to how the data herein has advanced the understanding of the potential ergogenic and adaptogenic role exogenous nucleotide supplementation may have followed high intensity exercise and to provide recommendations for future research based upon this body of work.

Realisation of aims and objectives

The primary aim of this thesis was to determine potential ergogenic, and adaptogenic role (s) exogenous nucleotide supplementation may have in healthy humans across a range of physiological systems following repeated sprint high intensity exercise.

The specific objectives of this thesis were:

1. To determine the dose response relationships of oral nucleotide supplementation at low, moderate, and high dose concentrations on repeated sprint performance (RSA), blood metabolites and hypothalamic pituitary adrenal axis (HPA) activation.
2. To determine the effect of chronic oral nucleotide supplementation at a high dose upon gastrointestinal permeability, damage, and symptoms blood metabolites and hypothalamic pituitary adrenal axis (HPA) activation following high intensity repeated sprinting exercise.
3. To determine the effect of 30 days oral nucleotide supplementation at high dose upon in vivo cell mediated immune responses using a skin contact hypersensitivity model and hypothalamic pituitary adrenal axis (HPA) activation following high intensity repeated sprinting exercise.

4. To investigate the effect of nucleotide supplementation on murine C₂C₁₂ myoblast cells and their impact on growth and differentiation in vitro in the absence or presence of inflammation induced via Tumour Necrosis Factor alpha (TNF- α) administration as an exercise mimetic.

Objective 1.

Objective one was addressed in Chapter 2. Chapter 2 was the first experimental chapter which was designed to investigate the effects of 30-days oral nucleotide supplementation at three dose levels designated (low [840mg], moderate [1680mg] and high dose [2520mg] concentrations) on repeated sprint ability (RSA) performance. The focus was to determine if nucleotides enhanced RSA performance and which dosing strategy of nucleotides provided the greatest enhancement in sprint performance. Seventy-four participants across a range of experimental designs, dose concentrations and duration completed the assessment of 4 sets of 6 x 35-meter RSA protocol with 15 second active recovery after completing 30-days oral supplementation. The main findings from Chapter 2 were that across several nucleotide dosing regimens no significant physiological effect and improvements in RSA performance compared to placebo were detected. The effectiveness of the present formulation of dietary nucleotides as an ergogenic aid that may enhance RSA high intensity exercise performance was not supported. It was envisaged that effects may therefore lie outside the RSA performance enhancement domain and instead be expressed as adaptogenic agents that facilitates the maintenance and enhancement of physiological system function, which express high cellular proliferative requirements and attenuates hypothalamic pituitary adrenal axis activation that exercise has been shown to affect.

Objective 2.

Objective two was addressed in Chapter 3. Chapter 3 was built directly upon the findings of Chapter 2 that reported no ergogenic effects of nucleotide ingestion but did not discount a putative adaptogenic role in impacting systems function. This chapter examined the functional adaptogenic role nucleotides may have on the gastrointestinal system which expresses elevated level of cellular proliferation and turnover and is shown to be adversely impacted by exercise

activity that damages gut barrier integrity increases permeability, induces cellular enterocyte damage, and increases subjective gastrointestinal symptomology expression. In this study, to determine effects upon gastrointestinal function, high dose [2520mg] nucleotides were supplemented orally for 30 days to investigate the gastrointestinal permeability, damage, and symptomology as well as effects on HPA activation following RSA activity. The principal findings show that barrier integrity as assessed by the appearance of lactulose and L-rhamnose in systemic circulation was not significantly altered, similarly enterocyte damage as measured by circulating IFABP concentration was unchanged together with no significant differences observed in subjective expression of gastrointestinal symptom following repeated sprint activity. Further, no observed effects on cortisol concentrations were apparent. These findings indicate that 30 days of high dose oral nucleotide (s) supplementation in recreationally trained males was ineffective in altering gastrointestinal function following high intensity repeated sprint exercise.

Objective 3.

Objective three was addressed in Chapter 4. Exercise disrupts innate and adaptive immune response altering both cell population numbers and functional ability. As a system that requires high rates of cellular proliferation, differentiation, and growth to meet potential antigenic challenge nucleotides are critical to ensuring that role is fulfilled. Immune system enhancements are frequently reported in human clinical and animal models where nucleotides are exogenously provided on a chronic basis. Adopting an in vivo cell mediated immunity approach using a skin contact hypersensitivity challenge present data considered the effect of 30 days high dose oral nucleotide supplementation upon the ability of the immune system to initiate and establish an integrated immune response that upon subsequent re-exposure would elicit an immune challenge to the initial antigen (DPCP) challenge in recreationally active young healthy males. The finding of this study indicates that in vivo immunity after 30-days oral nucleotide supplementation is not significantly improved relative to placebo treatments. In addition, no significant attenuations in cortisol release following exercise were also observed. Together the present oral nucleotide supplementation regimen expressed no significant immunological benefit or mediated a reduction in stress response using an in vivo immune model following high intensity repeated sprint exercise.

Objective 4.

Objective four was addressed in Chapter 5. Nucleotides are potent growth promoters and anti-inflammatory agents in animal models mediating significant increases in animal (carcass (muscle) mass), cell proliferation and reducing cytokine mediated inflammation, inflammation signalling pathways and promoting the release of anti-inflammatory mediators. The latter of these effects are important in muscle cell differentiation, and growth as evidenced in murine C₂C₁₂ myoblasts. This dual role of nucleotides as growth promoters and anti-inflammatory agents underscores their importance in maintaining cellular homeostasis and suggests their potential therapeutic applications in conditions involving inflammation and tissue damage. Thus, chapter 5 examined the effect of nucleotide supplementation on murine C₂C₁₂ myoblast cells and the impact on their growth and differentiation in vitro in both the absence or presence of a validated experimental induced cell stress model [inflammation via Tumour Necrosis Factor alpha (TNF- α) administration]. The findings of these in vitro experiments indicate that the addition of nucleotide to the growth media resulted in no significant difference in myoblast differentiation and myotube numbers and growth. In addition, whilst there was both enhancement and suppression of selected mRNA expression particularly TNF- α it was evident this did not translate through into impact on C₂C₁₂ differentiation and myotube morphology.

6.2 General Discussion

Adaptogenic and ergogenic ‘aids’ refer to substance(s) that enhance the body's ability to adapt to stress and improve physical performance. Ergogenic agents boost physical performance, endurance, and recovery by enhancing energy production, reducing fatigue, and improving muscle strength and function. (Brekhman et al., 1969; Bucci, 2022; Tulsawani and Vohora, 2023). Adaptogens help the body resist physical, chemical, and biological stressors by stabilising physiological processes and promoting homeostasis by regulating the body's response to stress (Marciniak et al., 2023). Nucleotides (NTs) are nitrogen-containing molecules consisting of a nitrogenous base (adenine, guanine, cytosine, thymine, or uracil), a five-carbon sugar (ribose or deoxyribose), and one or more phosphate groups (mono, di or tri) which it is suggested express such ergogenic and adaptogenic effects. Mechanistically, a pathway to such claims may exist. Nucleotides are integral to physiological functions in both humans and animals as components of the nucleic acids-DNA and RNA. They are essential for

cellular energy transfer, i.e., ATP (adenosine triphosphate). Nucleotides also participate in cellular signalling (e.g., cAMP/cGMP as a second messengers), enzyme regulation, and metabolic processes. Additionally, nucleotides are involved in the synthesis of metabolic coenzymes (e.g., NAD, FAD) and contribute to cell differentiation, growth, repair, and immune function; this breadth of functionality highlighting their critical importance in maintaining cellular and physiological functions (Carver et al., 1999; Grimble and Westwood, 2001; Hess and Greenberg, 2012; Ding et al., 2021; Abo-Sriea et al., 2024).

Given, importance of nucleotide(s) to multiple physiological pathways the principal findings of this thesis indicate no significant evidence that in healthy young male's chronic nucleotide (s) supplementation provides any ergogenic or adaptogenic effects following the imposition of an acute high intensity exercise model of stress. To consolidate these findings a synthesis model Figure 6.1 was developed to illustrate the observed in vivo and in vitro experimental outcomes derived from each of the individual chapters. Briefly, no significant improvements in RSA performance were evidenced in chapter 2, 3 and chapter 4 across a range of nucleotide dose and duration models. Similarly, in chapter 3 it was considered whether nucleotides could protect against exercise mediated gastrointestinal barrier dysfunction, again no significant adaptogenic effects were apparent regarding a reduction in gastrointestinal permeability, attenuation of apical enterocyte damage, and reduction in subjective gastrointestinal symptom expression. Likewise, in Chapter 4, the induction and elicitation of transcutaneous in vivo immunity following an RSA exercise challenge was unaffected between nucleotide and placebo loaded conditions. In all human RSA exercise protocols executed across Chapter 3, and 4, it was further apparent that attenuation of hypothalamic pituitary adrenal axis activation did not occur, indicating that systemic stress response (cortisol release) remained unchanged between nucleotide and placebo conditions. In chapter 5, an in vitro model of nucleotide(s) effects upon TNF- α induced stress in murine C₂C₁₂ myoblast cells (muscle cell growth, gene expression and anti-inflammatory action) showed no significant changes between nucleotide exposed relative to placebo cells upon myoblast differentiation, morphological change, and no consistent patterns of mRNA gene expression irrespective of nucleotide supplementation dose. Oral nucleotide supplementation remains unproven as conveying either ergogenic or adaptogenic role (s) during or following acute high intensity repeated sprint activity across a range of physiological systems in a healthy young male population.

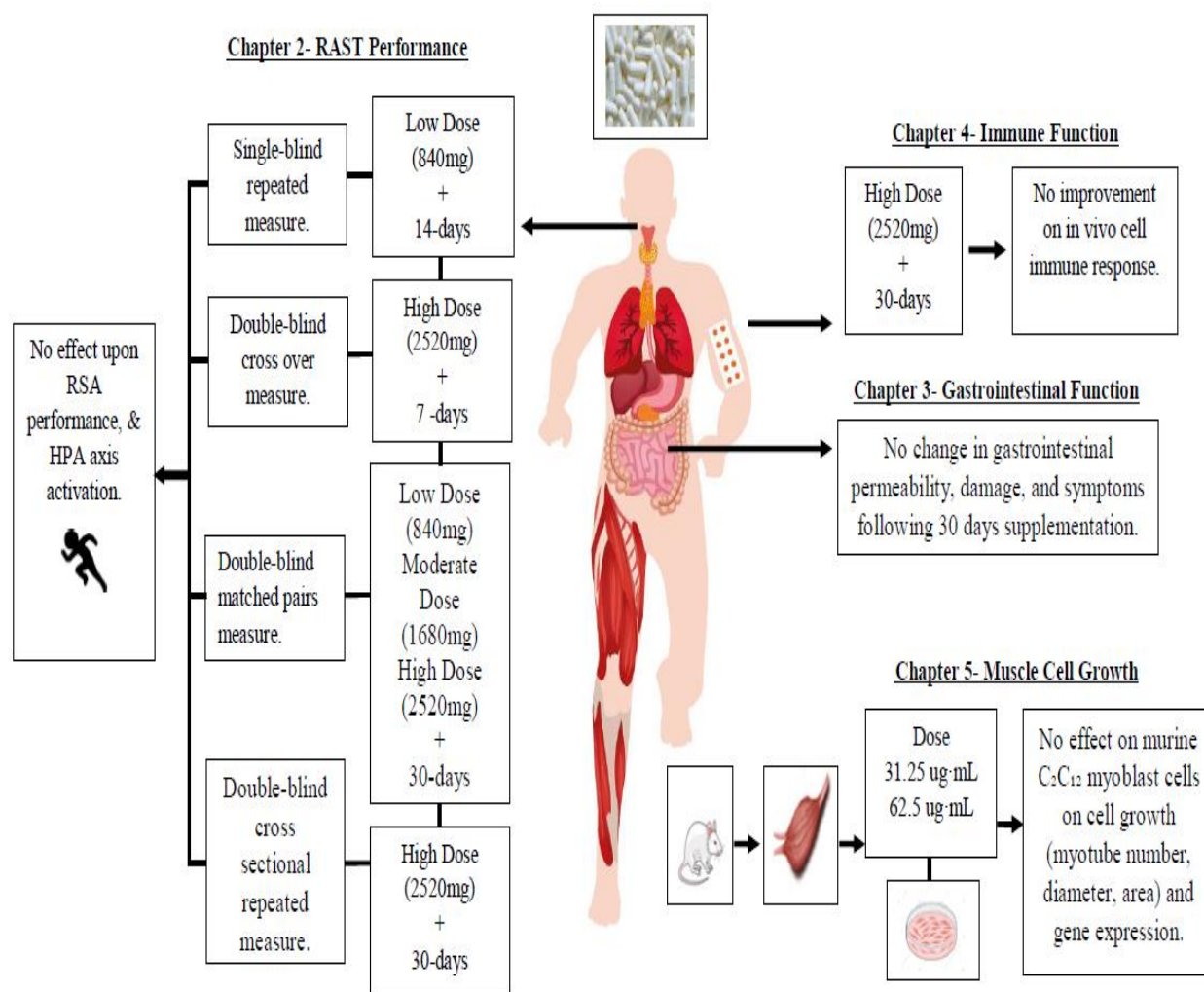


Figure 6.1. This synthesis model integrates findings from Chapters 2 through 5, assessing the effects of oral nucleotide supplementation on performance, gastrointestinal integrity, immune function, and cellular stress responses. No significant ergogenic or adaptogenic benefits were observed in repeated sprint activity (RSA) trials across various dosing and duration protocols. Similarly, nucleotide supplementation did not mitigate exercise-induced gastrointestinal permeability, enterocyte damage, or symptom expression. Immune assessments via transcutaneous challenge and systemic HPA axis activation (cortisol response) remained unaffected. In vitro analysis using C₂C₁₂ myoblasts exposed to TNF- α -induced stress showed no meaningful differences in differentiation, morphology, or gene expression. Collectively, the data suggest that oral nucleotides do not confer consistent.

This thesis examined efficacy of providing supplemental nucleotides on functional responses across several physiological systems, and as noted above in Figure 6.1 demonstrated no significant effects. In interpreting this data, the absence of nucleotide efficacy in this thesis could be because either there is no effect [but would be contrary to a body of evidence in neonatal/adult human clinical and animal literature which strongly suggest nucleotide(s) are bioactive and exert physiological functional changes] or the ‘sampling depth’ implemented in this thesis is inadequate to detect the nucleotide effect. The analogy here is deciding that the ocean has no fish simply because none are caught when a net is cast from shoreline. A clear ‘nucleotide paradox’ is therefore evident- an effect is expected but not detectable in this thesis. This may be due to the thoroughness of the sampling approach used. Therefore, is the ‘sampling depth’ in this thesis sufficient to detect an effect, especially when the nature and extent of nucleotide efficacy in humans in relation to exercise and non-clinical stress, is still poorly understood. Across each experimental chapter, several common thematic issues emerge which seem instrumental to examining the ‘sampling depth’ and the lack of nucleotides effectiveness observed across multiple physiological systems relative to placebo/control. These ‘sampling depth’ factors may broadly be categorised into the nucleotide(s) dose, formulation and bioavailability, the magnitude of the homeostatic challenge (physiological/stress load) imposed by the RSA exercise protocol, and TNF- α stress model and the interaction of these factors with the experimental population assessed (Figure 6.3, 6.4 and 6.5).

Most human and animal studies that have examined the efficacy of exogenous nucleotides have done so when administered orally. Central to our understanding as to why the present thesis shows no significant effects, and to the larger question as to whether nucleotide (s) ‘work’ (i.e., deliver ergogenic and adaptogenic effects when delivered orally), are several key questions: How much exogenous nucleotide should be ingested to deliver these effects? Of the amount ingested how much is delivered to where they are needed? What determines the final bioavailable amount present for nucleotide efficacy to be expressed? Regarding the first of these questions, guidance as to what constitutes the optimal exogenous nucleotide dose, composition, or duration of loading in adult humans is not established, with no definitive clinical or experimental data presenting a recommended amount. Recommendations are likely drawn from and reflect the examination of human dietary habits and estimated crude dietary nucleotide intake (Verkerk and Gil, 2009). A balanced human omnivorous diet is estimated to contribute somewhere between ~300-1500 mg·day of total crude nucleotide intake from nucleoproteins and nucleic acids (Suchner et al., 2001; Kaneko et al., 2014; Lockyer and

Stanner, 2016; Kaneko et al., 2020; Coelho et al., 2022; Wen et al., 2024). However, the absolute dietary nucleotide concentrations and relative purine to pyrimidine ratios delivered are highly variable and depend on how individual food(s) are grown, fed, processed, and stored in addition to population, cultural and individual factors which influence and prioritise food choices (Kaneko et al., 2020; Ding et al., 2021). Supplemental nucleotide dosing concentrations/recommendations ($\text{mg}\cdot\text{day}$) may reflect the attempt to redress the gap between what is considered an optimal dietary intake and what may now be ingested in the modern human diet. Commercially, this may also be reflected in the nucleotide supplements, currently marketed with recommended doses ranging from 100-500 mg, taken 1-3 times per day ($300\text{mg}-1500\text{ mg}\cdot\text{day}$) depending on the manufacturer and potentially the formulation. However, there is a lack of rationale and supporting evidence for these individual recommended dosing regimens.

This ambiguity is reflected in the wide range of dosing regimen applied in literature. In animal models where the biological effects of exogenous nucleotide are strongly demonstrated [such as improved exercise performance, exercise metabolism, immune function, anti-oxidant activity, anti-inflammatory activity, disease resistance, gut morphological development and muscle tissue differentiation and growth] nucleotides are typically administered orally at levels ranging from hundreds to thousands of milligrams of nucleotide per kilogram of body mass (Tahmasebi-Kohyani et al., 2011; Xu et al., 2017; Jang and Kim, 2019; Li et al., 2019; Perricone et al., 2019; Ridwanudin, et al., 2019; Ding et al., 2021; Dinardo et al., 2022). This ad-hoc range reflecting an approach based more upon what works in other models of stress rather than confirmed nucleotide turnover or requirement rates (Gasser et al., 1980; Grimble et al., 2000; Grimble and Westwood, 2001).

As a strategic approach, this thesis initially proceeded with the administration of low-moderate-high dose ($840\text{-}2520\text{ mg}\cdot\text{day}$) supplementation regimen (chapters 2) and high dose (Chapters 3 and 4) for several reasons. Firstly, the animal model work of Savaiano and Clifford (1978), Ho et al. (1979), Savaiano et al. (1980), Gasser et al. (1981) and human modelling of Coolen et al. (2011), broadly demonstrated only $\sim 2\text{-}10\%$ of orally ingested nucleotides could become bioavailable (i.e., 90-98% reduction). This supply side strategy reasoned that starting with a higher nucleotide dose would be crucial to compensate for this loss and maximise the bioavailable concentrations of nucleosides/nucleobases to be incorporated into nucleotide peripheral pools; thereby supporting the potential biological effectiveness of the

supplementation regimen. It was estimated that if at best ~10% of the 2520 mg dose (Chapters 2, 3, and 4) became bioavailable to peripheral nucleotide pools, this would still amount to ~252 mg of nucleotide (side) bioavailability. This potentially ‘bioavailable dose’ exceeds the ‘theoretical nucleotide bioavailability’ levels achieved from previous dosing strategies, where nucleotides have expressed positive effect upon human exercise performance, gastrointestinal function and immunity albeit with methodological caveats, as discussed in Chapters 2, 3, and 4. (Dancey et al., 2006; McNaughton et al., 2007; Riera et al., 2013; Casajus et al., 2014; Sterczala et al., 2016).

This dosing strategy whilst theoretically acting to maximise bioavailability would also remain considerably below the suggested ‘safe human’ acceptable daily intake (ADI) for purine nucleotides of ~4620 mg per day ($\sim 66 \text{ kg} \cdot \text{bw}^{-1}$) (Calloway, 1969; Verkerk and Gil, 2009). On an individual basis across all human experimental chapter’s, nucleotide(s) ingestion approximated $33\text{-mg} \cdot \text{kg} \cdot \text{bw}^{-1}$ [based on a 70 kg individual] under the present high dose protocol(s). In addition, when estimated contributions from dietary sources are factored in, total nucleotide intake is further elevated to $\sim 3000 \text{ mg} \cdot \text{day}$ ($\sim 42 \text{ mg} \cdot \text{kg} \cdot \text{bw}^{-1}$) across all participants. Whilst these estimates reflect general assumptions its cannot be discounted that individual nucleotide intake in this work may have varied both above and/or below this estimate. The supplementation regimen, in chapters [2, 3 and 4] represents the highest range of oral human nucleotide supplementation conducted to date in exercise studies but remain significantly lower than the doses used in animal experimental and habitual feeding studies. Previously, human nucleotide(s) intake per day of $\sim 50 \text{ mg}$ (Ostojic et al., 2013), 480 mg (McNaughton et al., 2007), 1440 mg (Riera et al., 2013), 600 mg (Casajus et al., 2014), 278 mg (Sterczala et al., 2016), 1500 mg (Dancey et al., 2006), with durations ranging from 14 days (Ostojic et al., 2013; Sterczala et al., 2016), 30 days (Riera et al., 2013; Casajus et al., 2014), to ~ 60 days (McNaughton et al., 2006; Dancey et al., 2006; McNaughton et al., 2007). By significantly increasing oral nucleotide ingestion beyond the dosing regimen reported in literature to deliver a bolus of nucleotides (840-2520 mg), it is clear the current supply side dosing strategies, or purine to pyrimidine nucleotide ratios ingested were systemically ineffective in producing positive changes compared to placebo in any of the primary outcome measures under the present RSA model of stress. Again, as noted in chapter 2, 3 and 4 this response or non-response phenotype exists as a function of potential individual participant designation as a nucleotide responder or non-responder predicated upon efficacy of their own endogenous supply pathways and exogenous nucleotide intakes.

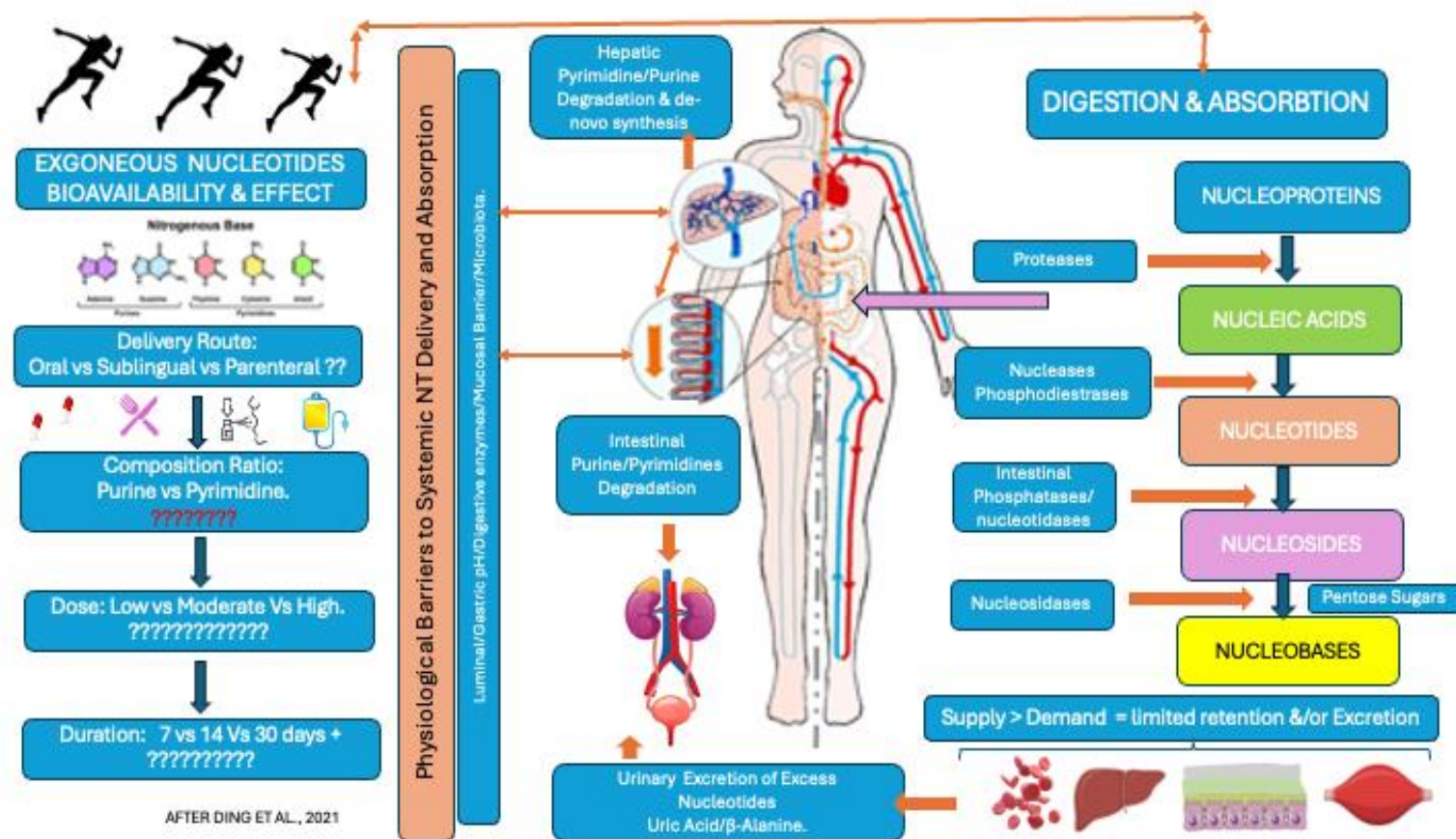


Figure 6.2. The bioavailability and fate of exogenous (orally/sublingual/parenteral) delivered nucleotides. The efficacy of nucleotides is dependent upon bioavailability. The route of delivery as either a refined supplement, complex food or via parenteral delivery pathways will impact bioavailability. Irrespective of purine to pyrimidine ratio, dose or duration of loading bioavailability is tightly regulated via several enteric and hepatic barriers. Paradoxically, orally ingested nucleotides absorbed as a refined supplement are not immediately bioavailable and must be degraded to enter and transit the physical gastrointestinal (enterocyte) barrier. Once entered into mesenteric circulation they transit to the liver and are further degraded and replaced with de novo synthesised output (Gasser et al., 1981; Savaiano et al., 1981).

Understanding the potential general (individual) dose-response relationships between ingested nucleotide concentrations, and the factors influencing bioavailability are, therefore, critical when considering this multi-system failure of nucleotides to elicit measurable physiological responses across chapters. In Figure 6.2, a summary of the potential factors influencing nucleotide bioavailability are presented. Firstly, a central paradox exists in understanding nucleotide dose-response outcomes in that, nucleotides when orally ingested are not ‘immediately bioavailable’ and cannot be used in their ingested form. Due to their structure, size, and hydrophobic nature, nucleotides are unable to pass through the enterocyte cell (gut) membrane. For absorption and bioavailability to occur, they must be degraded to their constituent parts (Weickman et al., 1981; Markiewicz, 1983) (Figure 6.2). This means that the nucleotides ingested are not the same nucleotides that might be eventually incorporated into peripheral and cellular nucleotide pools where they experience ‘90-98’% initial losses upon ingestion irrespective of the dose ingested. These losses primarily result from a step wise degradation process nucleotides undergo in the gastrointestinal tract. This process involves intestinal proteases, nucleotidases, and nucleosidases, which dephosphorylate nucleotides to nucleosides and inorganic phosphate followed by further breakdown of nucleosides into their component nucleobases and pentose sugars (Figure 6.2).

Subsequently, these nucleosides (nucleobases) are taken up through equilibrative, and concentrative nucleoside transporters located in the apical and basolateral membranes of gastrointestinal enterocytes (Rose and Coe, 2008; Bacha et al., 2013; Young et al., 2013; Pastor-Anglada and Pérez-Torras, 2018) (Figure 6.2). Savaiano et al. (1980) demonstrated a ten-fold reduction in nucleotide availability to peripheral tissues when orally administered (ingested) relative to parenterally (infused). Although more than 90% of dietary nucleotide degradation products i.e., nucleosides and nucleobases are absorbed during gastrointestinal transit, they are held ‘locally’ in the enterocytes and re-phosphorylated to nucleotides and never fully released into the circulation; being reused as part of an enterocyte nucleotide recycling process localised to the gut to meet the gastrointestinal tracts high cell proliferation and growth requirements (Figure 6.2). This local retention despite high dose delivery failed to translate into improved gastrointestinal function across both objective and subjective outcome markers in chapter 3 (Sonda and Tatibana, 1978; Cosgrove, 1995). An important caveat to consider in the biologically active pathway processes outlined above, is that across different participants the relative extent of each element may vary therefore altering potential individual bioavailability.

Sequestration of nucleotides(sides) to local enterocyte (gastrointestinal) tissue also means restriction of bioavailability to other peripheral nucleotide pools/tissue (Figure 6.2). Only 2-10% of nucleotides initially ingested are eventually incorporated into intra/extracellular nucleotide pools in a tissue and nucleobase specific manner (Savaiano and Clifford, 1978; Savaiano et al., 1980; Bronk and Hastewell, 1987; Uauy et al., 1994; Ho et al., 1997; Gimble and Westwood, 2001; Deng et al., 2017). The relative percentages sequestered may vary reflecting individual variations in enterocyte nucleotide needs. In this context, the gastrointestinal tract serves as an enteric barrier to regulate nucleotide absorption, retention and release of their degradation products predicated upon individual 'functional nucleotide needs' predicated by the level of stress experienced (Figure 6.3 and Figure 6.4). Further evidence of restricted nucleotide bioavailability to peripheral pools is provided by Gasser et al. (1981) who showed in rodents that bypassing the gastrointestinal (enteric) barrier (via infusion of isotope labelled plasma uridine directly into the hepatic portal vein) leads to rapid and extensive degradation of the labelled nucleotide (uridine) in a single circulatory pass through the liver. Critically, the labelled nucleotide is never re-released into the systemic circulation but is degraded and replaced through hepatic de novo synthesis, then released as unlabelled uridine at significantly lower concentrations. This suggests that the liver may also be acting as a 'control gate' in this thesis determining (possibly depending upon nucleotide demand) the level of nucleotide(s) released into systemic circulation regardless of the amount originally presented (Gasser et al., 1981; Gimble et al., 2000; Gimble and Westwood 2001) (Figure 6.2). However, hepatic removal process can be saturated when the initial dose exceeds processing capacity, leading to circulatory overflow and an increase in circulating physiological concentrations (Holstege, et al., 1992).

Supporting this latter view, Hanssen et al. (2023), demonstrated in humans that oral ingestion of supra-physiological doses (1000mg) of the pyrimidine nucleotide precursor (uridine monophosphate) as a single dose may acutely elevate circulating uridine levels outside the normal physiological range (~8uM) relative to lower dose (500mg), which do not (~4uM). Uridine levels return to physiological range within 120 minutes. This suggests that high oral dosing strategies adopted by this thesis could theoretically overwhelm this potential control gate/safety mechanism, leading to an oversupply of nucleotides relative to demand. However, whether the concentration of each nucleotide in the formulation was sufficient to exert such an outcome is undetermined. Xu et al. (2017) report that high dose nucleotide supplementation in mice impaired exercise performance and metabolism, compared to a lower, moderate dose.

Similarly, other animal and cell models have indicated comparable dose response profiles, where higher nucleotide doses lead to impaired functional outcomes. However, the threshold for what constitutes a "high dose" varies depending on the species, biological system, and form of stressor under investigation (Ohyanagi et al., 1989; Weaver and Kim, 2014; Hossain et al., 2018; Jang and Kim, 2019). This suggests that there may be a "sweet spot" dose for exogenous supplementation that is both situational and individually specific i.e. demand driven (Figure 6.3 and Figure 6.4). It may also be that the timing and distribution of the nucleotide ingestion impacts outcome; in so far as a high single dose may respond differently to lower doses (same absolute amount) delivered across several time points. The lack of circulating nucleotide levels in this work does not allow confirmation of this view. It would be a useful addition to present understanding to determine if nucleotides administered as either a bolus or distributed across a day elevated physiological ranges to the extent they impaired functional outcomes in humans.

The significance of these observations summarised in Figure 6.2 in the context of the present thesis, lies in the fact that the oral nucleotides ingested in Chapters 2, 3, and 4 are subject to enteric digestion, absorption, retention, release and subsequent hepatic processing, which together establish two distinct physiological barriers or "restriction points". These barriers form an "enteric/hepatic gateway" that regulates the bioavailability of any exogenously derived nucleotides (sides) to peripheral pools. In doing so, they maintain appropriate intracellular and extracellular nucleotide physiological ranges in response to current nucleotide pool demands (Gasser et al., 1981; Sanderson and He, 1994; Carver, 1999; Grimble and Westwood, 2001; Wurtman et al., 2000; Kilstrup et al., 2005; Waititu et al., 2017; Pastor-Anglada et al., 2018) (Figure 6.5). Additionally, ingested nucleotides (sides) that are not immediately dephosphorylated at the luminal gut brush borders and absorbed can become bioavailable to gut microbiota (Ding et al., 2022). These microbiotas may absorb and utilise the nucleotides for their own proliferation and growth, further restricting nucleotide bioavailability at the enteric barrier. In cases where large doses are ingested, as was done in this thesis, luminal nucleotide accumulation facilitating microbiota uptake may occur (Roman and Fitz, 1999; Waititu et al., 2017; Lee et al., 2020; Kasahara et al., 2023; Qu et al., 2023; Teng et al., 2023) (Figure 6.2.).

The maintenance of appropriate intracellular and extracellular nucleotide physiological ranges relative to current nucleotide pool demands (nucleotide homeostasis; functional nucleotide demand), is achieved through the precise control of nucleotide contributions from endogenous

sources [de novo and salvage biosynthetic pathways] and as noted above the control of exogenous [dietary and/or supplemental] nucleotide digestion, absorption, retention and the rate of nucleotide degradation (excretion) (Savaiano et al., 1980; Gasser et al., 1981; Rudolph, 1990; Lane and Fan 2015; Wang et al., 2016) (Figure 6.2; 6.3 and 6.4). Regulation of nucleotide physiological ranges is maintained by tissue specific enzymatic pathways that control nucleotide supply through feedback inhibition, where the end products of nucleotide metabolism act as allosteric inhibitors of key enzymes in the synthesis pathway(s) (Lane and Fan 2015). In purine salvage pathways, enzymes like hypoxanthine-guanine phosphoribosyltransferase (HGPRT) facilitate the conversion of purine bases into nucleotide monophosphates, utilizing PRPP (5-phosphoribosyl-1-pyrophosphate) as a precursor. This salvage pathway prevents unnecessary de novo synthesis of purine nucleotides. Similarly, in pyrimidine salvage pathways, enzymes like uracil phosphoribosyltransferase (UPRT) and thymidine kinase salvage pyrimidine bases (e.g., uracil, thymine) by attaching them to PRPP to form nucleotide monophosphates. Elevated levels of purine nucleotides can inhibit amidophosphoribosyltransferase (the first enzyme in purine synthesis), while elevated levels of pyrimidine nucleotides can inhibit carbamoyl phosphate synthetase II (the first enzyme in pyrimidine synthesis). Additionally, research has suggested that mTORC1, Akt and AMPK signalling could act to sense and regulate individual nucleotide(s) pool availability via de novo synthesis regulation (Ben-Shara et al., 2013; Zhu and Thompson, 2019; Shi et al., 2023).

These combined regulatory pathways, act to prevent overproduction or deficits ensuring optimal physiological concentrations of nucleotides are maintained by adjusting nucleotide flux through purine and pyrimidine biosynthetic and salvage pathways to meet functional nucleotide demands (Rudolph, 1990; Pedreño et al., 2012; Fridman et al., 2013; Lane and Fan, 2015; Buey et al., 2022). Simply increasing the exogenous nucleotide supply as undertaken in this thesis may not actually increase flux through to the nucleotides pools as its processing depends on multiple physiological, transport and enzymatic steps working in conjunction to handle the initial metabolite(s) concentration(s) (Thomas et al., 1998) (Figure 6.2). For instance, failure to regulate increases in adenosine and uridine concentrations can inhibit gluconeogenesis and impair hepatic glucose output, primarily through their actions as an allosteric inhibitor of phosphoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-bisphosphatase (FBPase), two key enzymes in the livers gluconeogenic pathway (Baker et al., 2010).

A further consequence of a failure to regulate excess nucleotide intake, is the potential risk of hyperuricemia particularly when the supplements are predominately purine based (Coleho et al., 2020; Coleho et al. 2022). However, analysis of the nucleotide doses administered in this thesis indicated no post-supplementation increases in circulating uric acid levels, with levels remaining within normal physiological ranges across all participants. This suggests that the purine content of the supplement, combined with any other dietary intake, was low enough to remain within clinical limits, indicating that enteric and hepatic pathways effectively processed (utilised) and/or excreted the available nucleotides, preventing excess uric acid accumulation (Figure 6.2). The uric acid profiles observed in all human experimental chapters (2, 3 and 4) align with the supplement's composition, which favours pyrimidine over purine nucleotides. The degradation products of pyrimidine catabolism [β -alanine, ammonia, carbon dioxide and acetyl-coenzyme A (CoA) and succinyl-CoA] and ribose or deoxyribose sugars, are considered relatively benign. No guidance on safe pyrimidine levels is included in acceptable daily intake (ADI) limits, as these products are rapidly used in the energy metabolism pathways; the tricarboxylic acid cycle and for gluconeogenesis, or quickly excreted via the liver and kidneys, depending on current biological needs (Moffatt and Ashihara, 2002; Verkerk and Gil 2009; Lane and Fan. 2015; Urasaki et al., 2016). Additionally, some metabolites, like β -alanine, may potentially benefit performance, although studies showing these effects typically involve higher doses (1.6 - 6.0 g·day). Whether β -alanine levels resulting from pyrimidine biodegradation in this work exert similar performance enhancing effects is undetermined.

In addition to higher nucleotide doses the type and ratio of purine to pyrimidine nucleotides incorporated in the formulation may be a critical factor influencing the physiological responses or lack thereof, observed across the chapters (Figure 6.2). Both in vivo and in vitro, Xu et al. (2017) and Uberti et al. (2020) observed in three different nucleotide formulations different levels of adaptation in mitochondrial enzyme concentration, energy metabolism, and antioxidant responses in rodent exercise and in vitro C₂C₁₂ myotube models. Uberti et al. (2020) reported that C₂C₁₂ myotube responses were highly dependent upon the composition of nucleotide mix utilised. A factor that may have influenced the lack of outcome seen in the present in vitro model of TNF alpha induced damage (Chapter 5). Similar responses have also been noted in animal models examining disease resistance (Hossain et al., 2018) where the presence or absence of different nucleotide resulted in varying levels of impaired immunity and mortality.

These findings support the concept that different purine and pyrimidine nucleotide formulation ratios may exhibit distinct roles that influence physiological pathways, depending on their relative concentration and the physiological systems being acted upon (Riera et al., 2013; Casajus et al., 2014; Deng et al., 2017; Uberti et al., 2020; Dihel et al., 2022; Hanssen et al., 2023). For example, in Chapter 2, where purine restoration following repeated sprint activity (RSA) is considered, the low-purine and high-pyrimidine ratio of the ingested supplements may not be optimal in this context. However, to the gut, immune and muscular system (Chapter 3, 4 and 5) pyrimidines are a vital component of cell replication and growth and would be expected to exert an influence something which was not observed in present data. Diehl et al. (2022) noted that excessive or imbalanced levels of individual nucleotides interfere/inhibit cell proliferation by disrupting the relative availability of nucleotide bases required for DNA replication, cell division, and growth. This suggests that the dosage and ratio of nucleotides provided in both the present in vivo and in vitro models may have unanticipated implications where they disrupt normal nucleotide balance (Figure 6.2 and Figure 6.3). In chapter 5 where in vitro C₂ C₁₂ cells were administered nucleotides little impact was apparent in their ability to significantly affect gene expression, cell morphological differentiation and growth potentially reflecting current nucleotide formulation specific effects that do not optimise these outcome markers; a finding that is in line with Uberti et al. (2020).

Currently, commercially available nucleotide formulations are optimised in different animal populations, to specific life cycle developmental stages to meet different physiological needs expressed during growth in those stages. For instance, in human premature infants and in piglets, the relative dose and formulation of nucleotides administered vary with age and level of imposed stress (Carver et al., 1999; Sauer et al., 2011; Jang and Kim, 2019). This indicates the necessity for formulation and supplementation strategies tailored to specific demands. Commercial marketing reflects this differentiation by targeting different nucleotide formulations to specific end-users in both animals and humans. However, use of a single nucleotide formulation in this thesis, despite three different target physiological systems, may have limited the potential for optimising the biological/physiological impacts. There may be a specific ‘Goldilocks [optimal]’ dose and formulation that is system-specific (e.g., optimal for the gut or skeletal muscle or immune system), suggesting that one ‘dose/ratio’ may not be suitable for all contexts and present thesis data could reflect this need for nucleotide dose and formulation specificity (Casajús et al., 2014; Uberti et al., 2020). Nevertheless, it is essential to consider that nucleotides play multiple functional roles, and inappropriate dosing or failure

to control nucleotide levels, or ratios could have serious adverse biological or clinical effects. Such dysregulation may impair RNA and DNA synthesis, leading to uncontrolled cell growth and proliferation (Ohyanagi et al., 1989; Idzko et al., 2014; Diehl et al., 2022; Shi et al., 2023; Tran et al., 2024). In Chapter 5 the implication of excess nucleotides was clearly expressed in the in vitro C₂ C₁₂ cell model, whereby the same nucleotide formulation as used in Chapters 2, 3, and 4 but at doses of 125, 250 and 500 µg·mL⁻¹ were toxic inducing a dose response elevation in cell death. However, lower doses of 31.25 and 62.5 µg·mL⁻¹ of nucleotides did not negatively impact on differentiation and myotube size with no ‘toxicity’ detected. Therefore, further consideration of these potential side effects in the context of the high-dose supplementation regimen used in this study is warranted (Figure 6.2 and Figure 6.4).

The critical question this thesis raises is why nucleotide efficacy was not observed across the various physiological systems examined in this thesis. These systems i.e., skeletal muscle, gut, and immune system all express high nucleotide turnover and thus requirement so as to maintain physiological function (Hess and Greenberg, 2012; Ding et al., 2021) Whilst the thesis adopted a ‘supply side model’ focusing on providing a high nucleotide dose in a standardised formulation, to improve bioavailability (subject to constraints noted previously), a key potential explanation for the lack of observed efficacy in this thesis could lie in the nature of the "demand signals" i.e., the ‘functional nucleotide demand’ imposed, as well as population-specific factors driving both the size and duration of these signals. These concepts can be resolved into a normal and high stress model (Figure 6.3 and 6.4 respectively).

A two-stage synthesis model (Figure 6.3 and 6.4) has been developed to reflect theoretically normal and high nucleotide demand scenarios. These figures visually map the key outcome domains; performance, gastrointestinal/cell repair integrity, immune function/metabolism, and cellular stress/signalling responses. They serve as an integrative framework, helping to visually contextualise the nucleotide responses across both in vivo and in vitro when a theoretical stress is applied where nucleotide demand is normal and met by mixed dietary and endogenous supply (Figure 6.3) and high where demand is not met by supply pathways (Figure 6.4).

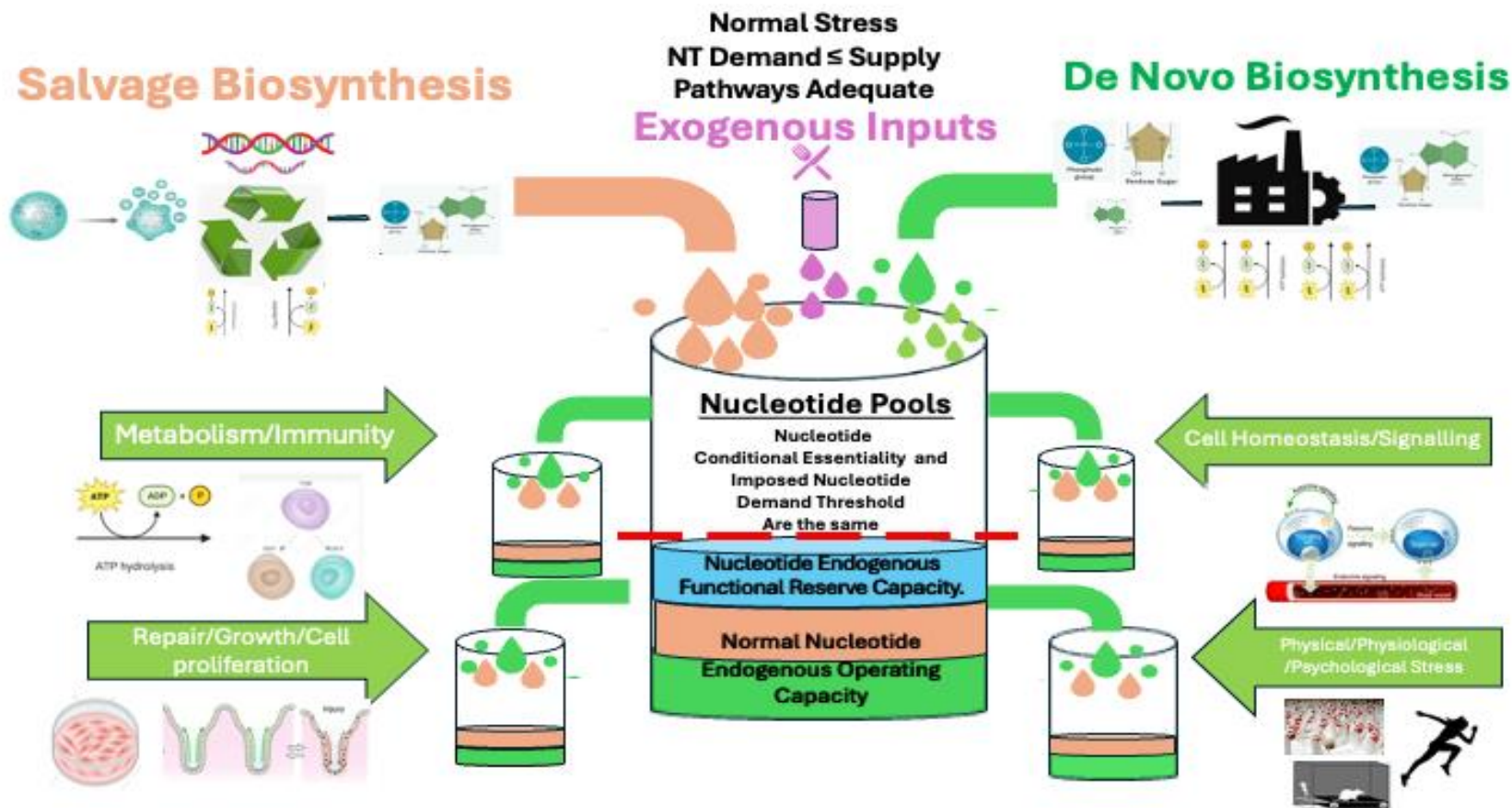


Figure 6.3. Schematic representation of nucleotide pathway contribution from both endogenous and exogenous pathways under normal/stress functional nucleotide demand scenarios. In the represented situation a balance between systemic demand and supply is shown where the combined requirements from peripheral nucleotides pools (Green arrows) is met from within the normal endogenous nucleotide supply operating capacity (Endogenous + Exogenous) with an inherent ability to upregulate nucleotide endogenous functional reserve capacity (output) to meet increased peripheral demand signal when required. In this model the nucleotide conditional essentiality threshold (Broken red line) is not exceeded, and present functional nucleotide demands are met without the need to provide supplemental nucleotides.

In both animal and human clinical models, where nucleotide efficacy is consistently demonstrated, including in physiological systems like those evaluated in this thesis, i.e., gastrointestinal, immune, skeletal muscle, the stress or ‘functional nucleotide demand’ signal is systemically and chronically elevated; nucleotide demand is therefore high (Figure 6.4) (Daly et al., 1995; Yau et al., 2003; Horvarth et al., 2023). These contrast the present thesis, where the ‘functional nucleotide demand’ signal triggered by the repeated sprint activity (RSA), was acute, transient, and arguably minor in nature when compared to the stress of other animal/human clinical models (Figure 6.3 and Figure 6.5). The critical factor that differentiates these models showing positive responses to exogenous nucleotide supplementation from those that do not (responsive vs non- responsive), appears to be the ‘functional nucleotide demand’ signal-specifically the magnitude and duration of the stress applied and its subsequent impact on peripheral nucleotide pool’s needs (Figure 6.3 [normal] vs Figure 6.4 [high]). Nucleotide supplementation appears to be most effective when the stress-induced demand exceeds the capacity of endogenous [de novo and salvage] pathway capacity to upregulate their activity to meet those demands i.e. high stress model (Figure 6.4).

Conceptually, the ‘functional nucleotide demand’ signal dictates whether exogenous dietary nucleotides reach a physiological threshold of conditional essentiality. This concept implies that nucleotides, are typically synthesised or salvaged endogenously in sufficient quantities to meet ‘normal physiological demands’ but become conditionally essential when the body’s endogenous biosynthetic and salvage capacity is exceeded thus requiring additional exogenous provision (Carver and Walker, 1994; Van Buren and Rudolph, 1997; Carver, 1999; Gimble and Westwood, 2001). In a nucleotide homeostatic model, stress increases nucleotide requirements (i.e., functional nucleotide demand) which disrupts system nucleotide balance leading to an adaptive response to return the system back toward a functional equilibrium (Copper, 2008; Billman 2020). In this thesis, RSA-induced stress caused an acute disruption in systemic homeostasis due to increased cellular bioenergetic demands, purine metabolite degradation and efflux, heightened immune cell recruitment and activation and, the need to maintain gastrointestinal barrier function through enterocyte differentiation, growth, and repair (Figure 6.5). To restore nucleotide homeostatic balance during RSA challenge, endogenous salvage and biosynthetic pathways must upregulate, shifting from an initial "supply set point" (Set Point 1) to a higher "supply set point" (Set Point 2) to meet the imposed ‘functional nucleotide demand’ of the stress applied (Figure 6.3 and Figure 6.5).

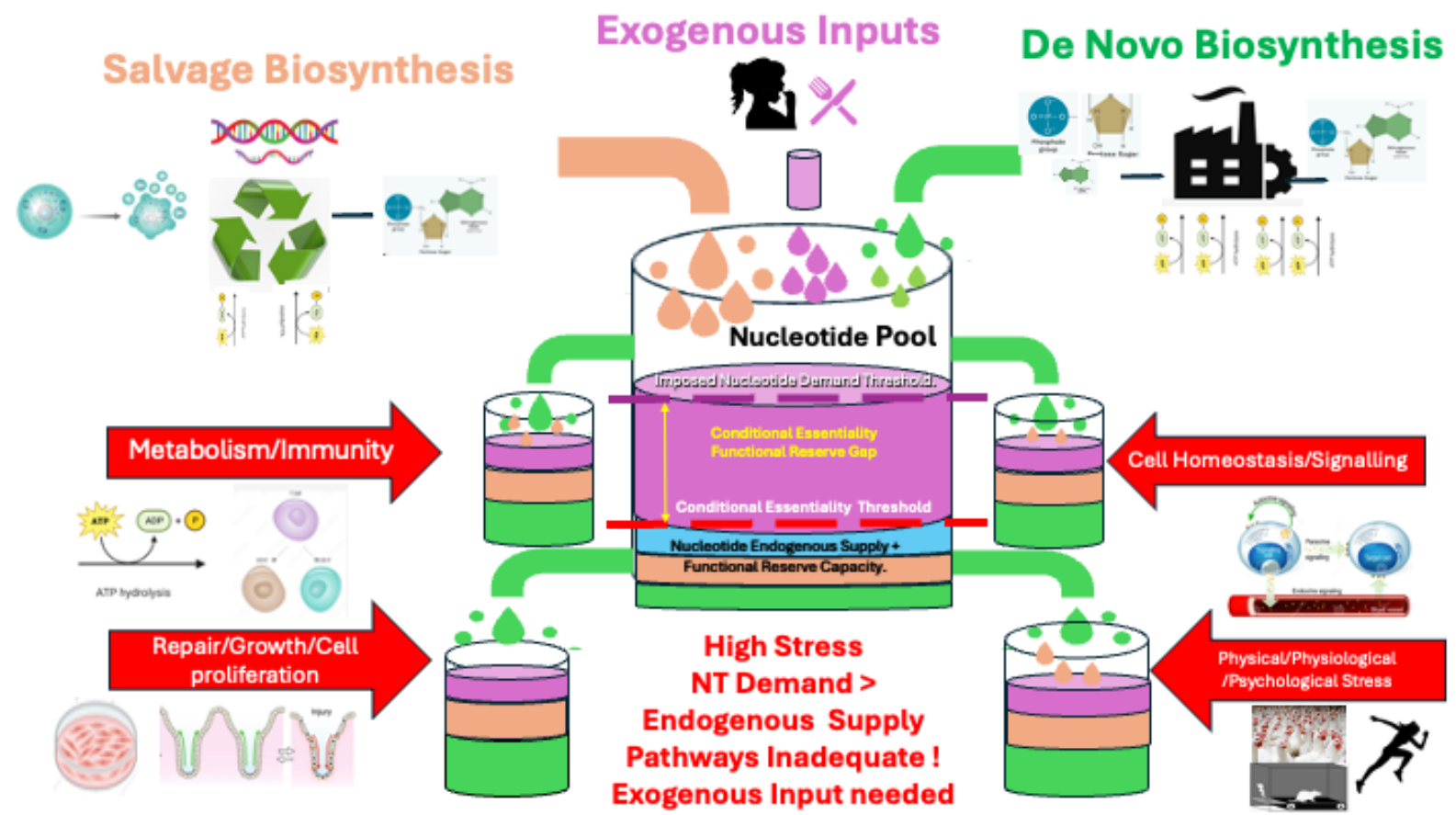


Figure 6.4. Schematic representation of nucleotide pathway contribution from both endogenous and exogenous pathways under high stress situations. In the represented situation there is an imbalance with systemic nucleotide demand elevated above supply. The combined requirements from peripheral nucleotides pools (Red arrows) are not met from within the normal endogenous operating capacity (Endogenous + Exogenous), the functional nucleotide reserve whilst upregulated are inadequate and unable to sufficiently meet increased demand. The nucleotide conditional essentially threshold is breached (Broken red line) and the Conditional Essentiality Functional Reserve Gap (Δ endogenous supply – functional nucleotide demand) must be filled with supplemental exogenous nucleotides.

The demand signals that set the 'functional nucleotide demand' are not isolated but rather governed by each individual's physiological status in relation to their maximal capacity to respond to stress. Healthy individuals following an omnivorous diet typically exhibit efficient nucleotide biosynthesis and salvage pathways, allowing them to support nucleotide homeostasis. In this context, these systems retain a 'nucleotide functional reserve capacity' i.e., an ability to upregulate these endogenous supply pathways when subjected to increased stress or demand (Figure 6.5). The magnitude of the nucleotide functional reserve capacity, and its maximal capacity to respond [hypothetically measured by its 'Fractional utilisation rate' relative to the imposed 'Functional Nucleotide Demand'] is likely influenced by each participant's prior exposure to that stress which may lead to chronic systemic adaptations (training) to that stress moderating its potential effects on nucleotide demand. Currently, there is limited data on how nucleotide metabolic pathways within specific compartments respond. For example, the gastrointestinal and immune systems indicate that it is plausible that training-related adaptations occur, as evidenced by changes in other morphological and functional markers of these systems (Kurowski, 2022; Wallet et al., 2022;), these adaptations have yet to be quantified for nucleotide metabolic pathways.

In the context of the present thesis participants presented with a broad range of, physical activity and sport related backgrounds, functional aerobic capacities, habitual training status, training specificity particularly in relation to sprint/RSA exercise. This heterogeneity in participant characteristics, combined with the uneven distribution of these traits across experimental groups, may have introduced variability, potentially confounding the relationships between nucleotide demand and the capacity of salvage and biosynthetic pathways to meet that demand. There is substantial support for factors influencing the functional demand for nucleotides and the subsequent fractional utilisation of the 'functional nucleotide reserve capacity' to meet this demand. For instance, Skotnicka et al. (2010) reported reductions in purine nucleotide loss following high intensity exercise in highly trained relative to recreationally trained males. Similarly, Zielinski et al. (2013) described training specific adaptations in nucleotide metabolism, which include lower purine metabolite loss and improved purine salvage responses through higher nucleotide enzyme pathway activity, distinguishing the superior system capacity of sprint-trained from endurance-trained athletes. Zielinski et al. (2019) and Pospieszna et al. (2019) further demonstrated in endurance trained relative to untrained individuals across a broad age range (20-90 years), improved purine metabolism, attenuated purine loss and more efficient purine pool recovery after exercise.

Pospieszna et al. (2021) and Kistner et al. (2021) also confirmed both a hierarchy in adenine nucleotide metabolism relative to training specificity [sprint trained > endurance trained > active > untrained] and improved nucleotide metabolism response to habitual training. This variability is not limited to inter-individual differences but can also occur within individuals, reflecting tissue-specific nucleotide demands and prioritisation of supply. For example, immune tissues may take precedence over muscle during disease (e.g., immune > gut > muscle), while gut ischemia prioritises the gut over immune and muscle tissues (e.g., gut > immune > muscle) and exercise prioritises skeletal muscle (e.g., muscle > gut > immune) although how this is achieved complex and still poorly defined (Cosgrove, 1995; Hess and Greenberg, 2012; Gimble and Westwood, 2001; Ding et al., 2021).

Collectively, these findings suggest population-specific differences in nucleotide metabolism, driven by general or specific training related adaptive responses, which enable individuals to effectively conserve and/or restore nucleotide loss. Such differences underscore the importance of considering participant heterogeneity when evaluating the efficacy of nucleotide responses. Given that the imposed RSA stress is likely to challenge endogenous nucleotide salvage and biosynthesis pathways differently, each participant may rely on varying proportions of their functional nucleotide reserve capacity to meet the imposed functional nucleotide demands. Consequently, it is essential to carefully consider the heterogeneity in participant characteristics and their distribution across groups. Acknowledging this variability is crucial for interpreting observed outcomes in performance, as well as gastrointestinal and immune responses, relative to these potential confounders (Figure 6.5).

Considering, the performance data collected across all exercise chapters implies that the RSA demand signal and resultant ‘functional nucleotide demand,’ likely remained within the functional nucleotide reserve capacity of each participants nucleotide biosynthesis and salvage pathways. These allowed participants to meet the imposed challenge (Figure 6.3 and 6.5). These findings further reflect the inherent flexibility and robustness of the endogenous nucleotide biosynthesis and salvage pathways, enabling systemic supply to be adjusted to either whole-body or local demand without recourse to exogenous nucleotide provision. This adjustment maintains tight physiological control of intracellular and extracellular nucleotide pools, as observed in non-human models (Gasser et al., 1980; Taut, 1994; Yamamoto et al., 2011; Fridman et al., 2013; Zhu and Thompson 2019; Dihel et al., 2022; Kim-Holzapfel et al., 2023). A key implication, therefore, is that the RSA exercise model employed throughout this

thesis was not sufficiently intense or prolonged to disrupt nucleotide homeostasis to a degree where the ‘functional nucleotide demand’ exceeded the systems redundancy across a diverse participant range (Figure 6.5). This lack of disruption prevented the detection of potential nucleotide efficacy, as evidenced by the failure to differentiate outcome responses in high-nucleotide-demand systems (immune, gastrointestinal, and skeletal muscle) between nucleotide-supplemented and supplemented trial (Figure 6.5).

These observations suggest that large deviations from participants' normal physiological nucleotide demands [likely over extended periods] would be required for nucleotide biosynthetic and salvage pathways to become constrained in their response capacity, potentially breaching their conditional essentiality threshold. In such scenarios, exogenous nucleotides could transition into "conditionally essential" nutrients, resulting in a functional reserve gap between what is needed and what can be supplied (Cosgrove et al., 1995; Van Buren and Rudolph, 1997; Grimble and Westwood, 2001) (Figure 6.4 and 6.5). The observed deficit between the required and available nucleotides suggests that exogenous nucleotides may be necessary to fill this potential gap, optimise nucleotide pool supply, and support or restore functional responses, as demonstrated in high-stress animal and human models (Kulkarni et al., 1986; Carver and Walker, 1995; Cosgrove et al., 1996; Carver, 1999; Yau et al., 2003; Sauer et al., 2011; Xu et al., 2017; Hossain et al., 2018; Horvath et al., 2023; Nakagawara et al., 2023) (Figure 6.5).

Such perspectives are supported by findings from Andrés-Elias et al. (2007) and Sauer et al. (2012) who have demonstrated that in a ‘porcine model’ that exogenous nucleotides are only ‘conditionally essential’ for piglet health and growth when habitually when exposed to adverse housing conditions, while no such effects were observed under optimal housing conditions. Similarly, exogenous nucleotides supplementation has been shown to improve a range of biological markers in avian and aquaculture models only under conditions of chronic high stress, not low stress (Burrells et al., 2001; Hossain et al., 2016; Hossain et al., 2018). This suggests that the “functional nucleotide demand signal” imposed by the physiological stress ‘is ‘a’ if not ‘the’ critical driver for the level of nucleotide supply, whether through endogenous or exogenous pathways, and determine the need to resort to exogenous sources to meet increased demands (Figure 6.3, 6.4 and 6.5).

Elite athletes and recreational “weekend warrior” athletes, who often face multiple stress inputs [such as high training volumes and intensities, inadequate recovery, monotonous training regimes, dietary restrictions (e.g., low-nucleotide vegan or vegetarian diets), inadequate sleep, and psychological or lifestyle stressors] could present a scenario where endogenous nucleotide pathways fail to upregulate sufficiently to meet demand (Figure 6.5). These continuum models of high-volume/intensity training, overreaching, and overtraining [potentially combined with syndromes such as relative energy deficiency in sport (RED-S) (Langbein et al., 2021; Stellingwerff, 2021)] may offer a setting where nucleotide efficacy in human sport models could be demonstrated, with ‘functional nucleotide demand’ been elevated beyond ‘functional reserve capacity’ resulting in the conditional essentiality threshold been breached and a ‘conditional essentiality functional reserve gap’ opening up between nucleotide requirement and endogenous supply (Figure 6.5). Where exercise models have shown potential nucleotide efficacy particularly in athlete models, they are likely subject to sustained training/habitual stress (Riera et al., 2013; Casajus et al., 2014; Sterczala et al., 2016). This scenario would parallel the chronic high-stress conditions seen in animal models, where nucleotide efficacy has been repeatedly demonstrated (Andrés-Elias et al., 2007; Sauer et al., 2012; Xu et al., 2017).

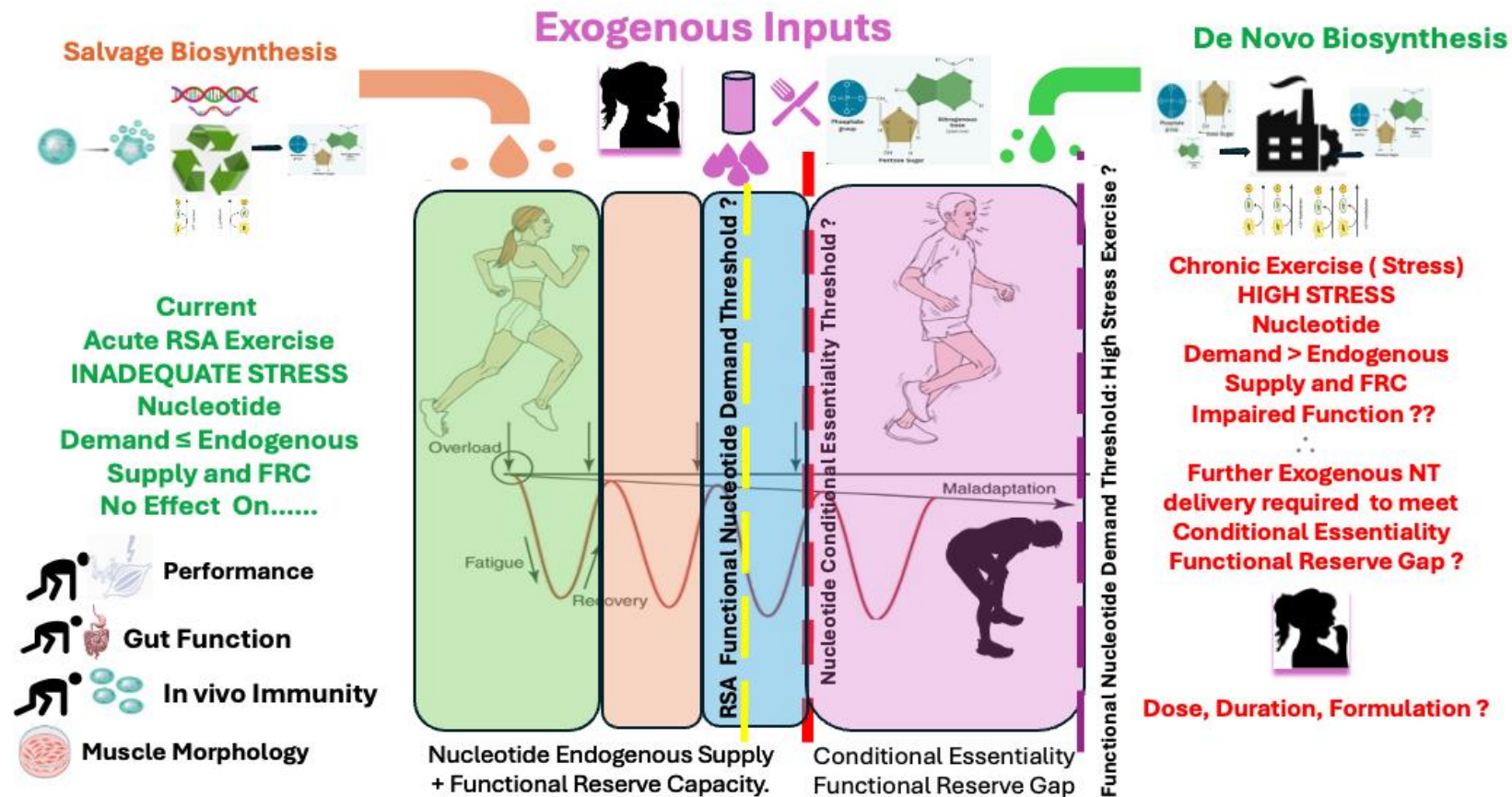


Figure 6.5. Schematic representation of nucleotide pathway contribution derived from both endogenous and exogenous pathways under current RSA exercise stress and postulated high stress exercise scenarios. In this model combined habitual and RSA exercise stress imposes a functional nucleotide demand (FND) [Broken yellow line] that is adequately met from within current nucleotide supply [endogenous + dietary+ functional reserve capacity pathways] which theoretically is less than or equals the Nucleotide Conditional Essentiality Threshold (NCET) [Broken red line] a threshold that represents the combined capacity of current nucleotide supply mechanisms to upregulate their activity and meet FND. In such a scenario additional exogenous nucleotides are not required. Alternatively, when training stress load increases transitioning from an adaptation to maladaptation (high stress) model the NCET is breached, endogenous + dietary + functional reserve capacity pathways cannot supply sufficient nucleotide(s) relative to imposed FND [Broken Purple Line] and a Conditional Essentiality Functional Reserve Gap opens. Under high stress situations this nucleotide deficit can only be met through increasing inputs from exogenous sources either dietary and/or supplemental.

6.3 Thesis Conclusions

Nucleotides play critical roles in a wide array of biological processes; however, their demand can fluctuate significantly raising the potential that exogenous sources may be required to address shortfalls in the body's endogenous capacity to meet these needs during periods of elevated stress, particularly exercise. This thesis explored whether supplementing with high dose exogenous dietary nucleotides could support various physiological functions [like sprint (exercise) performance, gastrointestinal health, immune function, and muscle cell growth] during periods of acute high-intensity exercise stress. Despite, previous studies in animals and certain clinical models having suggested that nucleotide supplementation may enhance these functions, especially under stress, present thesis findings, do not support the hypothesis that exogenous nucleotides provide ergogenic (performance-enhancing) or adaptogenic (stress-adapting) benefits. Any potential benefits of exogenous nucleotides, previously observed in other high stress models, were not translated and expressed under the exercise and in vitro stress conditions applied in the current thesis (Figure 6.1).

Explanation for the lack of observed benefits is likely tied to the physiological mechanisms regulating nucleotide availability and demand. Unlike other nutrients that can be readily absorbed and utilised, dietary nucleotides undergo extensive degradation and reassembly before they can be incorporated and used based on the body's specific needs. The gastrointestinal tract and liver serve as regulatory "control gates," limiting the immediate availability of nucleotides in response to systemic/peripheral pool functional demand, rather than merely increasing levels in response to an elevation in supply. This control process ensures nucleotide levels remain stable, preventing excess intake from overwhelming both local and systemic nucleotide homeostasis disrupting cellular/physiological functions (Figure 6.2).

The high doses of exogenous nucleotides, administered in this thesis to maximise bioavailability through a supply side delivery model, appear to have encountered these inherent biological safety mechanisms and was unlikely to have succeed, because the corresponding functional nucleotide demand signal from the RSA exercise remained too low to necessitate significant uptake of these exogenous nucleotides (Figure 6.3). This indicates that exogenous nucleotide supplementation may have limited value for recreational athletes, under typical exercise conditions of low to moderate RSA exercise stress (i.e., a normal demand signal model), as the body appears adequately capable of meeting nucleotide demands endogenously. It may be as

outlined a high stress exercise model mimicking that seen in high stress animal models is required to fully interrogate nucleotide efficacy (Figure 6.4) Further research is needed to explore alternative models of exercise stress, dosing, formulation and athletic populations, to determine if any potential nucleotide benefits can be unlocked (Figure 6.5).

6.4 Recommendations

Despite several studies investigating the effects of dietary nucleotides on exercise performance, the exact mechanisms by which nucleotides express those effects particularly how they absorbed and become bioavailable remain poorly understood in humans. It remains unclear if dietary nucleotide can reach the target systems outlined in this thesis effectively. Current evidence suggests that dietary nucleotides are broken down into nucleosides and nucleobases during digestion before being absorbed in the small intestine. However, the rate of absorption, the influence of gut microbiota, and the role of specific transporters in humans are still not fully determined. The optimal dosing regimen in terms of amount, formulation and method of nucleotide delivery needed to optimise bioavailability are yet undetermined. Further research is therefore needed to explore the pathways involved in this bioavailability delivery pathway and the nucleotide distribution process to target tissues, particularly the skeletal muscle, immune system, and gut. Answers may be possible through measuring nucleotide turnover in humans using stable isotopes, particularly through a method known as isotopic labelling. This technique involves administering labelled nucleotides, which can be incorporated into newly synthesised nucleic acids. Once the labelled nucleotides are introduced, biological samples (e.g., blood, tissue biopsies) can be collected at various time points to determine their rate of absorption. Mass spectrometry can be employed to detect and quantify the isotopic composition of nucleotides within these samples through analysing the ratio of labelled to unlabelled nucleotides, it would allow determination of the rate of nucleotide turnover at rest and /or during or after exercise models. This approach will provide key insights into the bioavailability of nucleotides and cellular metabolism, cell proliferation, and the dynamics of nucleotide pool turnover in different target physiological systems, across different exercise intensities, modalities, and populations.

Much of the existing literature on the ergogenic and adaptogenic effects of dietary nucleotides is based on studies in animals, which limit the ability to translate conclusions directly to humans. The knowledge derived around the short and long-term effects of exogenous nucleotide supplementation is more extensive in porcine, aquaculture, bovine and avian models and

provides more detailed understanding of both cellular and whole system adaptations than that determined in humans; this knowledge gap needs to be addressed. While nucleotides have shown benefits in infant growth and immune function, there is a lack of longitudinal studies assessing their impact over extended periods across a range of physiological systems. Most human nucleotide exercise studies extend for a maximum duration of up to 60 days. Conducting long-term, randomised controlled trials (RCTs) in diverse human exercising and non-exercising populations, including different age groups and performance status, is essential to assess the safety, ergogenic and adaptogenic efficacy, and potential side effects of nucleotide supplementation. Combinations of methodological approaches identified above will facilitate this process. These trials as with the current thesis should examine human based endpoints like those outlined in this thesis. Specifically, exogenous nucleotides express immunomodulatory properties, particularly in enhancing both innate and adaptative immune responses and reducing inflammation. Further research should focus on combination in vivo and vitro models to examine which immunological cellular and humoral elements are impacted, the molecular basis of that change and which specific nucleotide formulation and dose act to modulate immune responses in exercising humans.

Emerging evidence also suggests that dietary nucleotides may play a role in shaping the composition of the gut microbiota, which is linked to various performance and health outcomes. While some animal studies have reported changes in microbial populations in response to nucleotide supplementation, human research is needed to clarify these effects given the role the enteric barrier expresses in regulating the uptake, retention, and release of orally ingested nucleotides. Specifically, future studies should employ microbiome sequencing techniques to investigate how nucleotides influence microbial diversity and whether these changes mediate physiological benefits, such as improved gut function, enhanced immunity, improved muscle metabolism or reduced inflammation and how this again links to nucleotide dose and formulation.

Finally, nucleotides, as fundamental building blocks of nucleic acids, play a crucial role in cellular energy metabolism, particularly in the context of mitochondrial biogenesis and function. Nucleotides are thought to enhance mitochondrial efficiency by supporting ATP synthesis through their actions on coenzymes in the electron transport chain (ETC) impacting cellular respiration. Since mitochondria are central to oxidative metabolism, examining muscle $\dot{V}O_2$ can offer insights into mitochondrial function changes, particularly in response to nucleotide intake.

However, no animal or human research have considered mitochondria-related effects of oral nucleotides during or after exercise. Near-infrared spectroscopy (NIRS)-derived muscle oxygen consumption ($m \dot{V}O_2$) could provide a mechanism for investigating these mitochondrial related changes following dietary nucleotide supplementation. NIRS provides non-invasive, real-time monitoring of muscle tissue oxygenation and $m \dot{V}O_2$ by measuring changes in haemoglobin and myoglobin saturation. This contrasts more invasive biopsy approaches to assess muscle mitochondria number volume and enzymatic changes. This could help determine whether nucleotides improve mitochondrial function, potentially enhancing endurance, recovery, or muscle performance, and provide a non-invasive way to track bioenergetic adaptations over time.

Whilst dietary nucleotides supplementation could hold promise for promoting ergogenic and adaptogenic effects in humans many gaps remain in our understanding of their precise effects, the above recommendations address potential avenues for consideration around nucleotide supplementation in humans, however many other potential physiological avenues exist and merit further exploration.

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